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SHORT COMMUNICATION
Effects of anti-odor automobile air-conditioning system products on adherence of *Serratia marcescens* to aluminum

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Sixteen commercial products for use in automobile air-conditioning systems (ACS), most designated for abatement of malodors presumably of microbial origin, were examined for their potential to inhibit attachment and to detach cells of the Gram-negative bacterium *Serratia marcescens* on aluminum sections. Numbers of attached cells were appreciably reduced (>60%) following immersion in three alcohol-type and two acrylic-coating-type products. Several products had essentially no effect on the attached cells. Most of the products indicated for alleviation of associated microbial odors from ACS provided only short-term effects. When products were coated onto aluminum prior to exposure to the cells, water-insoluble coatings appeared to provide more consistent inhibition of primary adherence of *S. marcescens*. The differences in degrees of primary adherence of a selected strain of *S. marcescens* to variously treated aluminum provided a rapid and reproducible assessment of potential antimicrobial efficacy of ACS products.
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Introduction

Automobile air-conditioning systems (ACS) with their concomitant condensation of moisture from air may provide environments for the growth of microorganisms. A fleeting and sometimes persistent malodor may be the only sign of these microbes but allergic reactions may occur [2–4]. Kumar *et al* [4] reported that 18% of 224 patients with symptoms of allergic rhinitis or asthma suffered exacerbation of their symptoms after use of their ACS. Twenty-two of 25 cars of these patients yielded various fungi and actinomycetes but "no correlation was observed between the make and model or year of the automobile, the type of organism found, or the presence or absence of exacerbations of allergic rhinitis or bronchial asthma." These investigators had earlier associated a case of hypersensitivity pneumonitis to exposure to a thermophilic actinomycete contaminant of an ACS [3]. Kumar *et al* [2] removed, cleaned and returned the air conditioner evaporator cores of four automobiles. The types of fungi isolated from the ACS before this undertaking were isolated again from the systems 3 weeks later. Washing the evaporator, however, was reported to lower significantly the densities of fungi isolated from the air-conditioning vents.

Research in our laboratories has shown that a broad range of bacteria, protozoa and fungi can colonize various components within the ACS [7–9]. Retention of moisture within the ACS in conjunction with the growth of microorganisms such as *Methylobacterium* and *Penicillium* spp. have been implicated as major causes for the malodor in passenger compartments of cars. The growth of microorganisms in ACS occurred in both new and used vehicles [7].

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Commercial products are now available in both the US and abroad that are specifically marketed for treatment of malodors, presumably of microbial origin, emanating from ACS. Many of the available products are recommended for repeated applications (as needed) due to recurrence of the problem. Repeated applications and particularly replacement of the evaporator and other components involve considerable expense and inconvenience for the consumer. Therefore, selection of an appropriate treatment product for the "sour" odors produced by microorganisms is important. Herein we report on the use of a strain of *Serratia marcescens* in a primary adhesion or adherence test [1] for comparing relative antimicrobial potentials of various ACS treatment products to prevent initial irreversible attachment and to reduce numbers of cells already attached to aluminum.

Materials and methods

Aluminum sections (10^2 mm) were rinsed with 70% ethanol to remove any oils from handling and twice with 0.9% sterile saline (ss) to remove residual alcohol. *S. marcescens* ATCC 13880 was selected for its optimal adherence to aluminum from a screening of six isolates. The adherence tests were adapted from a procedure described by Ahearn *et al* [1]. Briefly, an overnight culture was harvested by centrifugation, washed with ss, suspended in minimal medium containing $\sim 2 \times 10^8$ cells/ml (as determined by spectroscopy) and incubated for 1 h at 37°C and 120 rpm in an Innova 4080 rotary shaker incubator (New Brunswick Scientific, Edison, NJ). L-[3,4,5-^{3}H]leucine (specific activity 150 Ci/mM; NEN, Boston, MA) was added and incubation continued for an additional 20 min. The radiolabeled cells were harvested by centrifugation, washed 3× with ss and suspended in phosphate-buffered saline to a cell density of 10^8 cells/ml. Each product and

Table 1 Effect of various air-conditioning treatment products on relative primary adherence of *S. marcescens* on aluminum sections

Code	CFU[a]	Percent reduction	CFU	Percent reduction	Composition[b]
	Immersion[c]		Coated[d]		
A	215±20	59	345±21	30	unk
B	511±46	3	417±38	16	unk
C	542±38	0	201±25	59	ni
D	359±49	32	262±45	47	unk
E	221±14	58	289±46	42	unk
F	295±29	44	455±30	8	citrus, bu, pr
H	189±19	64	380±44	23	ald
I	222±29	58	415±41	16	unk
J	163±33	68	652±60	0	alc, qac, bu, pr
K	21±2	96	–[e]	–	apa
L	22±2	96	–	–	apa
M	464±25	12	904±147	0	alc
N	267±18	49	–	–	alc, qac
O	465±19	12	–	–	alc, bu, pr
P	422±22	20	–	–	alc, phen, bu, pr
Control	526±26	0	495±59	0	sal

[a]Number of colony-forming units (cfu) per section ($\times 10^4$) ± standard error; $n = 5-10$.
[b]Apa, acrylic base-phosphated amine; alc, alcohol; ald, aldehyde; bu, butane; ni, nonionic surfactant; phen, substituted phenol; pr, propane; qac, quaternary ammonium compound, sal, saline; unk, unknown.
[c]Aluminum sections (10^2 mm) exposed to 3×10^8 cells for 2 h. Sections with adhered cells were rinsed and immersed in 1.0 ml of product for 10 min.
[d]Aluminum sections (10^2 mm) were immersed in products, rinsed and immediately exposed to 3×10^8 cells radiolabeled with leucine.
[e]Not determined; in case of K and L, film not produced without drying step.

the control (untreated aluminum) for each test series contained five replicates.

The radiolabeled cell suspension was dispensed into individual wells of Costar 24-well cell cluster plates (Corning, Corning, NY) that contained aluminum sections and then incubated for 2 h at 37°C (120 rpm). At 2 h, cells were mostly attached singly with occasional small clumps [1]. The irreversibly bound cells that were retained on the substratum (aluminum) after a standard rinsing procedure (see next section) were defined under "primary adherence." The aluminum sections with adhered cells were rinsed with ss and placed into cell cluster plates that contained 1.0 ml of the various ACS treatment products for 10 min at 37°C at 120 rpm (Table 1).

Primary adherence to coated aluminum

To study adherence to coated aluminum, sections were immersed in 1.0 ml of product for 10 min, rinsed 5× in three separate volumes of 180 ml ss and transferred to 3.0 ml of a suspension of cells radiolabeled with leucine. In a second coated-test series, the aluminum sections were removed from the treatment products and dried for 2- and 40-day periods at 45°C (Table 2). These dried and aged sections were rinsed in six separate volumes of ss before their addition to a suspension of leucine-labeled cells.

The sections with adhered cells from all test series were rinsed in ss and transferred to Opti-Fluor scintillation cocktail (Packard, Meriden, CT), and disintegrations per minute were measured by an LS 6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA). Disintegrations per minute (adjusted for background radiation on nonradiolabeled aluminum) were converted to colony forming units with a calibration curve produced from plate counts of serial dilutions of the radiolabeled cell suspension. Data analyses were performed with Sigma Plot 4.0 (Jandell Scientific, Sausalito, CA).

Results

The primary adherence of *S. marcescens* on aluminum sections was reduced upon their immersion in most of the products, but only four (K = L > J > H) gave greater than 60% reductions (Table 1). These four products when coated onto aluminum prior to exposure to the cells did not necessarily reduce primary adherence to coated aluminum in a similar relative order to the immersion data

Table 2 Relative primary adherence of *S. marcescens* to aluminum sections coated with various air-conditioning treatment products[a]

Code	CFU[b]	Percent reduction	CFU	Percent reduction
	Aged 2 days[c]		Aged 40 days[d]	
C	82±29	53	118±9	33
D	150±22	15	101±9	43
E	235±35	0	254±25	0
K	18±7	89	53±7	70
L	29±5	84	93±12	48
P	219±35	0	344±35	0
Control	207±28	0	177±29	0

[a]Representative products were coated onto aluminum and rinsed and heat-aged at 45°C before exposure to cells.
[b]Number of cfu per section ($\times 10^4$) ± standard error; $n = 5-10$.
[c]Treated aluminum was aged for 2 days.
[d]Treated aluminum was aged for 40 days.

(Table 1). Products K and L did not produce a uniform and dry film within the time for the manipulation of the test samples in this test series. Primary adherence to coated aluminum appeared unaffected or exceeded that for the controls with several products that reduced primary adherence with the immersion procedure (J and M, Table 1). Activity of one product, C, was anomalous in that no activity was observed following immersion of the adhered cells, but primary adherence of the cells to aluminum coated with C was reduced (59%).

Representative products dried and aged on aluminum for 2 and 40 days and rinsed prior to exposure to radiolabeled cells were compared for residual anti-adherence activity (Table 2). Cells were prepared as per the standard procedure but different inocula preparations were used for the different aging periods. Product (E) representing an alcohol-solvent-type lost activity with aging, whereas another product (D) showed enhanced activity. Products K and L retained the highest degrees of activity with aging.

Discussion

Labels of several of the products examined in this study claimed an antimicrobial effect, but most, particularly those sold in the US, were simply indicated for treatment of odors and for repeat application as required. All of the products were designed for spraying into a working ACS, but modes of application differed. Three products (K, L and P) were indicated for direct application to the evaporator core, whereas others were directed to the air intakes. Our *in vitro* test protocol involved an immersion in an equivalent volume of the materials because we had no information on concentrations of active ingredients, and we attempted to develop a uniform quantitative procedure. Although it is possible that certain propellants may act to enhance activity, this was not noted in this study. Product M, which contained alcohol with a pump delivery system, and O, which contained alcohol plus butane/propane propellants, gave insignificant reduction of primary adherence, i.e., irreversibly bound cells.

Mariscal *et al* [5] observed residual antimicrobial activity of a glass surface following exposure to a water-soluble chlorinated disinfectant but not alcohols. Four of the products tested in this study showed some degree of residual efficacy on aluminum against adherence of *S. marcescens*; two of these products, K and L, were determined the most effective. Both K and L contained a phosphated-amine antimicrobial of low water solubility reported earlier to be active when bound into a plastic matrix [6]. These two products were also the most active when an accelerated aging process was applied to the coated samples. The heat applied in this aging process probably contributed to some variability in activities (e.g., L) and apparent enhancement (e.g., C) by altering or concentrating the active compounds. The data also suggest that certain water-soluble products, particularly alcohol-based types, designated for malodors in ACS have at best short terms of activity, and some products may even stimulate the primary adherence of *S. marcescens*.

The procedures described herein provide a relatively rapid quantitative method for assessing at least one parameter of antimicrobial activity, inhibition of primary adherence. The protocol is a good way to evaluate relative efficacies of products in a test series when they are compared with the same inoculum and a standard control. Most products studied made no antimicrobial claim and we did not evaluate their capacity to mask or reduce odors. Further comparisons of our test protocol with *in situ* observations of ACS are in progress for establishment of the appropriateness of the inhibition of primary adherence of *S. marcescens* as an acceptable procedure for evaluation of ACS products.

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