



## Elucidation of bacteria found in car interiors and strategies to reduce the presence of potential pathogens

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The human microbiome is influenced by a number of factors, including environmental exposure to microbes. Because many humans spend a large amount of time in built environments, it can be expected that the microbial ecology of these environments will influence the human microbiome. In an attempt to further understand the microbial ecology of built environments, the microbiota of car interiors was analyzed using culture dependent and culture independent methods. While it was found that the number and type of bacteria varied widely among the cars and sites tested, *Staphylococcus* and *Propionibacterium* were nearly always the dominant genera found at the locations sampled. Because *Staphylococcus* is of particular concern to human health, the characteristics of this genus found in car interiors were investigated. *Staphylococcus epidermidis*, *S. aureus*, and *S. warnerii* were the most prevalent staphylococcal species found, and 22.6% of *S. aureus* strains isolated from shared community vehicles were resistant to methicillin. The reduction in the prevalence of pathogenic bacteria in cars by using silver-based antimicrobial surface coatings was also evaluated. Coatings containing 5% silver ion additives were applied to steering wheels, placed in cars for five months and were found to eliminate the presence of culturable pathogenic bacteria recovered from these sites relative to controls. Together, these results provide new insight into the microbiota found in an important built environment, the automobile, and potential strategies for controlling the presence of human pathogens.

**Keywords:** *Staphylococcus*; microbial ecology of built environments; fomite; antimicrobial coatings

### Introduction

The environments that humans encounter daily are sources of exposure to microbial communities and some are of potential concern to human health. Humans spend a significant amount of time indoors and thus the microbial ecology of indoor environments likely impacts the human microbiome (Klepeis et al. 2001; Kembel et al. 2012). In the past few years studies have emerged that examine the microbial communities of built environments. Current evidence suggests that indoor microbiomes originate mainly from outside air or the human skin (Pakarinen et al. 2008; Rintala et al. 2008; Grice & Segre 2011).

The microbial species present in a built environment are predominantly determined by exposure to microbes and selection of certain microbial types by the environment (Martiny et al. 2006). Previously, most studies examining the microbiome of built environments have concentrated on buildings, with a particular focus on health care settings (Rintala et al. 2008; Tringe et al. 2008; Amend et al. 2010; Kembel et al. 2012). In this study, both culture-dependent and culture-independent approaches were utilized to study the microbial ecology of the automobile built environment. Automobiles are potentially important fomites for exposure to microbes as

many individuals spend a significant amount of time in this setting.

The *Staphylococcus* genus is of particular importance concerning fomite colonization and transmission to humans. Staphylococci frequently colonize human skin and mucosal surfaces, and thus are likely to be transmitted to inanimate surfaces that humans come into contact with (Safdar & Bradley 2008; Foster 2009; Pynnonen et al. 2011; Payne et al. 2013). Of particular concern is *Staphylococcus aureus*, which has the capacity to cause a variety of devastating infectious diseases (Lowy 1998; Klevens et al. 2007; Otto 2012). *S. aureus* infections and outbreaks have previously been associated with exposures to a multitude of contaminated fomites including whirlpools, razors, towels, handrails and toys (Miller & Diep 2008; Kassem 2011). Because staphylococci can colonize commonly touched inanimate objects, it is feasible that interior surfaces of automobiles could serve as reservoirs for pathogenic staphylococci and may play an important role in human colonization and infection.

This study addresses three general questions. First, what is the relative abundance of microbes at different frequently touched automobile surface interiors? Second,

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what is the composition of the microbial communities found in automobile interiors? Third, can automobile interior surfaces be designed to resist colonization by potential pathogens?

## Materials and methods

### *Sample collection and bacterial culturing*

Samples were collected from indicated automobile interior sites using Whatman neutralizing buffer swabs (GE Healthcare Life Sciences, Piscataway, NJ, USA). These swabs were wetted in the provided storage/transport buffer and  $\sim 6.5 \text{ cm}^2$  of the indicated surface was sampled. The swabs were suspended in 2 ml of sterile phosphate buffer saline (PBS) and vortexed for 30 s to re-suspend the bacteria. For culturing, suspended samples were serially diluted in sterile PBS to extinction. Dilutions were plated on nutrient agar (Becton Dickinson, Franklin Lakes, NJ, USA) and TSA blood agar (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 3 days at 30°C.

### *Bacterial isolate identification via partial 16S rRNA PCR amplification and sequencing*

For 16S rRNA sequencing, single colonies were suspended in 100  $\mu\text{l}$  of sterile water and heated to 100°C for 15 min. The suspension was centrifuged for 10 s at 6,400 rpm and 5  $\mu\text{l}$  of each suspension were used as the template for PCR amplification of the 16S rRNA gene. PCR was performed using a mix of 25  $\mu\text{l}$  of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 18  $\mu\text{l}$  of PCR certified water (Promega), and 1  $\mu\text{l}$  each of forward primer (8FPL, AGTTTGATCCTGGCTCAG) and reverse primer (806R, GGACTACCAGGTATCTAAT) as previously described (Vornhagen et al. 2013). PCR products were cleaned using the QIAquick PCR Purification System (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The purified DNA was checked for quantity and purity using a NanoQuant Tecan M200 (Tecan, Durham, NC, USA). Sequencing was performed by the DNA Sequencing Core at the University of Michigan (Ann Arbor, MI, USA) using Applied Biosystems 3730xl DNA Analyzers (Applied Biosystems, Carlsbad, CA, USA), BigDye3.1 chemistry (MCLAB, San Francisco, CA, USA), and the protocols recommended by the manufacturer. Resulting partial 16s rRNA gene sequences were analyzed using CHROMAS (Brisbane, Australia) and compared to known sequences in the National Center for Biotechnology (NCBI) database using the Basic Local Alignment Search Tool (BLAST).

### *Culture-independent analysis*

For culture-independent analysis, car interiors were sampled as described above and swab tips were removed

and placed in sterile 2.0 ml vials. Samples were stored at  $-80^\circ\text{C}$  prior to analysis by pyrosequencing. DNA was extracted using a modified Qiagen DNA preparation kit, which included incubating the swab in lysis buffer for 1 h, bead-beating utilizing a Qiagen Tissue Lyser, and column capture, purification and elution of DNA. PCR was conducted to generate barcoded amplicons with linkers. To prepare for FLX sequencing, the size and concentration of DNA fragments were determined by using DNA chips within a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Hercules, CA, USA) and a TBS-380 Fluorometer (Promega Corporation, Madison, WI, USA). A sample containing  $9.6 \times 10^6$  molecules  $\mu\text{l}^{-1}$  of double-stranded DNA with an average size of 625 bp was mixed with 9.6 million DNA capture beads and subsequently amplified by emulsion PCR. After bead recovery and enrichment, the bead-attached DNAs were denatured with NaOH, and sequencing primers were annealed. A two-region 454 sequencing run was performed on a  $70 \times 75$  GS PicoTiterPlate using a Genome Sequencer FLX System (Roche, Nutley, NJ, USA). Following sequencing, all failed sequence reads, low quality sequence ends (Avg Q25), short reads  $< 150$  bp (final mean length 412 bp) and tags and primers were removed. Sequence collections were then depleted of any non-bacterial sequences, sequences with ambiguous base calls, sequences with homopolymers  $> 5$  bp in length, and chimeras as previously described (Bailey et al. 2010; Callaway et al. 2010; Capone et al. 2011; Handl et al. 2011). To determine the predicted identity of microorganisms in the remaining sequences, they were de-noised, de-replicated, and OTU clustering was performed using uClust ([www.drive5.com](http://www.drive5.com)). Sequences were then queried using BLASTn against a highly curated custom database of high quality 16s bacterial sequences derived and manually curated from NCBI. Using a NET analysis pipeline, the resulting BLASTn outputs were compiled and data reduction analysis was performed as described previously (Bailey et al. 2010; Callaway et al. 2010; Handl et al. 2011; Capone et al. 2011). Bacteria were classified at the closest well-characterized genus. Rarefaction analysis was conducted using QIIME (Caporaso et al. 2010). Sequences were de-noised and chimeras removed using UCHIME (Edgar et al. 2011). Sequences  $< 250$  bp were removed and sequences  $> 250$  bp were trimmed to 250 bp. Sequences were then normalized to 1,200 bp and 10 iterations of rarefaction were performed to evaluate the number of species present.

### *Measurements of antibacterial activity on surfaces*

To determine the ability of *S. aureus* (strain ATCC6538P) to colonize different engineered car surfaces, the procedure outlined in the Japanese International Standard

for Measurement of antibacterial activity on plastic surfaces, JIS Z 2801, was utilized. Briefly, two surfaces were examined: a polycarbonate/ABS (Sabic Cycoloy MC8002, Ford Motor Company, Dearborn, MI, USA) hard plastic surface with the same formulation used to mold many interior car parts and a soft polyurethane foam surface used to cover steering wheels. The PC/ABS substrata were coated with a black, two-component, solvent borne urethane coating (Product 318LE/303LE, Red Spot Paint, Evansville, IN, USA) designed for interior automotive trim. The polyurethane foam substrates were coated with a 1-component waterborne coating (Red Spot Paint, product 458 W) using an in-mold process typically used for automotive steering wheels. In addition to a control formulation of each of the above coatings, which contained no antimicrobial additives, four formulations of each coating were prepared with the addition of antimicrobial additives to the liquid coatings described above (458 W and 318LE/303LE). These coatings contained: 3% Agion silver ion (Sciessent, Wakefield, MA, USA), 5% Agion silver ion, 1% micronized polyolefin wax coated with nanosilver (Deurex MXAg 9,520, Deurex AG, Germany), and 4% silane quaternary ammonium salt (Biosafe HM4100, Biosafe, Pittsburgh, PA, USA). Each formulation was sprayed on the respective substratum at a thickness of  $\sim 25 \mu\text{m}$ . After coating, the panels were cut into  $50 \times 50 \text{ mm}$  squares and sterilized by submerging in 70% EtOH. *S. aureus* test inoculum of  $6 \times 10^5$  was generated by suspending bacteria in dilute nutrient broth (1:500) and 400  $\mu\text{l}$  of the suspension were placed on the plastic surfaces that were placed in the Petri dish. The surfaces were covered with a sterile piece of stomacher bag ( $40 \times 40 \text{ mm}$ ) that spread the inoculum evenly over the surface. Samples were incubated at  $35^\circ\text{C}$  at a relative humidity  $> 90\%$  for 24 h. Bacteria were recovered from the plastic surfaces by adding 10 ml of SCDLP broth ( $17 \text{ g l}^{-1}$  casein peptone,  $3 \text{ g l}^{-1}$  soybean peptone,  $5 \text{ g l}^{-1}$  NaCl,  $2.5 \text{ g l}^{-1}$   $\text{Na}_2\text{HPO}_4$ ,  $2.5 \text{ g l}^{-1}$  glucose,  $1 \text{ g l}^{-1}$  lecithin,  $7 \text{ g l}^{-1}$  polyoxyethylene sorbitan monooleate) to the plastic surface and vigorously pipetting up and down to remove attached bacteria. The number of viable colony-forming units (CFUs) remaining on the surfaces was determined by plating serial dilutions in nutrient agar, incubating for 48 h at  $35^\circ\text{C}$  and counting colonies.

To assess the long-term efficacy of the coatings formulated to resist microbial colonization, some coated materials were aged *via* an artificial accelerated weathering protocol, SAE J2412. During the test, surface specimen exposures were monitored based on measured light at  $1.06 \text{ W m}^{-2}$  (@ 420 nm) using a lamp and filter combination that closely mimics the wavelength distribution of light transmitted through automotive glass. Specimens were cycled between a 1 h dark cycle at  $38^\circ\text{C}$  and 95% humidity and a 3 h light cycle at  $70^\circ\text{C}$  and 50%

humidity. Surface specimens were removed after exposures of  $2,500 \text{ kJ m}^{-2}$  and  $5,000 \text{ kJ m}^{-2}$  for evaluation.

### Antimicrobial susceptibility testing

All *S. aureus* isolates were tested for susceptibility to the antimicrobials listed in Table 3 using the Clinical and Lab Standards Institute broth microdilution method (Bou 2007). Mueller–Hinton broth was purchased from BDD and MIC plates were incubated at  $35^\circ\text{C}$  for 24 h. *S. aureus* strains ATCC 25,923 and ATCC 29,213 were utilized as control strains for each antimicrobial susceptibility assay.

### Statistical analyses

Statistical analyses were performed using a 1-way analysis of variance (ANOVA). Results are expressed as mean  $\pm$  standard error (SE) of the mean, unless otherwise indicated.

## Results

### Relative abundance of colony-forming units at interior car locations

To gain insight into relative abundance of bacterial colonization in different areas of car interiors which might serve as fomites, locations that were reasoned to come into frequent contact with the driver were swabbed (indicated in Figure 1A). A total of 18 cars from the Ford Motor Company employee fleet were swabbed. Analysis of colony-forming units (CFUs) from each site revealed that each swabbed location had culturable bacteria present and that there was significant variation in total CFUs in each location from car to car (Figure 1B). However, by this analysis, the most highly colonized locations with over 100 culturable CFUs per  $6.5 \text{ cm}^2$  surface area were areas of frequent touching by the occupants, including locations on the steering wheel (A, K), the gear shifter (C), door handles and window switches (E, G), and the center console near the beverage holder (D).

### Culture independent analysis of bacteria in car interiors

To gain insight into the types of bacteria present on highly colonized areas culture independent analysis was conducted. Steering wheels (A), gear shifters (C), and the center console (D) were swabbed in five cars (car numbers: 19, 21, 22, 23, 24) and extracted DNA was subjected to bTEFAP FLX massively parallel pyrosequencing. Each sampled site possessed a unique bacterial community at the genus level (Table 1). The most dominant bacterial genera that were present at all sampled sites were *Staphylococcus* and *Propionibacterium*.



**B**

	A	B	C	D	E	F	G	H	I	J	K
Car 1	172	20	79	349	12	27	20	55	6	ns	ns
Car 2	22	24	61	40	11	16	8	19	2	ns	ns
Car 3	100	28	44	351	311	14	11	9	7	ns	ns
Car 4	536	205	175	660	465	ns	ns	ns	ns	ns	ns
Car 5	53	ns	30	340	ns	ns	ns	ns	ns	11	15
Car 6	200	110	120	25	55	ns	ns	ns	ns	ns	ns
Car 7	155	145	115	501	ns	ns	ns	ns	ns	ns	330
Car 8	330	155	14	185	40	ns	ns	ns	ns	ns	ns
Car 9	55	130	435	265	ns	ns	ns	ns	ns	ns	40
Car 10	50	ns	130	180	ns	ns	ns	ns	ns	105	55
Car 11	215	ns	300	145	ns	ns	ns	ns	ns	45	1445
Car 12	295	ns	35	1070	10	9	920	ns	5	ns	ns
Car 13	1665	ns	35	480	1370	ns	ns	15	ns	ns	ns
Car 14	135	ns	ns	50	ns	ns	ns	ns	ns	ns	ns
Car 15	102	80	ns	25	30	ns	35	ns	ns	ns	ns
Car 16	55	ns	70	2705	ns	7	60	ns	2	15	442
Car 17	70	ns	85	340	ns	ns	ns	ns	ns	135	290
Car 18	95	ns	ns	1408	ns	11	226	50	ns	105	502
<b>Ave:</b>	<b>239</b>	<b>99</b>	<b>115</b>	<b>506</b>	<b>256</b>	<b>14</b>	<b>182</b>	<b>29</b>	<b>4</b>	<b>69</b>	<b>390</b>
	+/-378	+/-65	+/-114	+/-658	+/-448	+/-7	+/-333	+/-21	+/-2	+/-52	+/-465

Figure 1. Analysis of CFUs present at different car interior locations. (A) Image of a typical car interior with swab sampling sites labels. Swabs of  $6.5 \times 6.5$  cm area were collected from (A) steering wheel, (B) radio volume knob, (C) gear shifter, (D) center console, (E) door latch, (F) door lock, (G) door lock control, (H) door handle, (I) window control, (J) cruise control button, and (K) interior steering wheel. (B) Number of CFUs isolated from swab locations A–K from 18 different cars. ns = not sampled.

Genus-level differences extended to the level of bacterial class and upon averaging across samples from the five cars, 38.3% of the sequences derived from bTEFAP were members of the class Bacilli (car 19: 52.1%, car

21: 56.0%, car 22: 55.9%, car 23: 7.4%, car 24: 20.3%); 21.4% were members of the class Actinobacteria (car 19: 28.5%, car 21: 10%, car 22: 15.9%, car 23: 25.1%, car 24: 27.2%), 10.9% were Gammaproteobacteria (car 19:

Table 1. Estimated relative abundance of bacterial genera (%) from swabs of the indicated car locations as determined by bTEFAP.

Genus	19A	19C	19D	21C	21D	22C	22D	23A	23C	23D	24A	24C	24D
<i>Staphylococcus</i>	77.1	74.1	10.2	19.1	3.32	14.0	63.7	12.5	10.5	1.05	19.7	29.2	28.2
<i>Propionibacterium</i>	9.72	17.5	3.5	7.44	0.50	6.04	2.75	22.6	8.53	.06	21.3	17.4	0.08
<i>Pantoea</i>	1.21	0	0	0	0.07	0	0	5.01	0	98.2	0	0	0
<i>Exiguobacterium</i>	0	0	0	0	91.8	0	0	0	.03	0	0	0	0
<i>Acidovorax</i>	1.23	0.17	6.55	3.41	0	9.22	0.02	7.55	3.75	0	11.5	2.96	23.5
<i>Streptococcus</i>	1.02	0.07	4.17	7.30	0	12.5	5.49	5.80	0	0.06	9.93	13.2	5.15
<i>Clostridium</i>	0.15	1.16	0	14.3	0	3.00	0.11	0.23	13.1	0.03	1.79	5.67	1.93
<i>Mycobacterium</i>	0.22	0.01	0.39	0	0	0.09	0.12	0	38.0	.05	0	0	0.13
<i>Massilia</i>	0	0	0	14.3	0.04	1.83	0	0	0	0.04	0.45	0	8.45
<i>Acinetobacter</i>	0.64	1.32	0.57	1.00	0	2.61	0	1.66	0.21	0.01	9.06	1.48	3.22
<i>Micromonospora</i>	0.05	0	19.0	0	0.03	0	0.24	0.07	0	0	0	0	0
<i>Sphingomonas</i>	0	0	5.89	0	0.05	4.99	0	0	1.72	0.02	3.02	0	2.92
<i>Corynebacterium</i>	0.61	0	0	2.49	0	0.19	0	4.03	0	0	7.06	6.89	1.42
<i>Dehalococcoides</i>	0.07	0	10.4	0	0	0	0	5.71	0	0	0	0	0
<i>Brevundimonas</i>	0	0	0	0	0	0	0	12.5	0	0	0	0	0
<i>Gemella</i>	0.01	0	0	0	0	7.10	0.24	0	0	0.01	0	5.21	0
<i>Trichococcus</i>	0	0	0	0	0	12.1	0	0	0	0	0	0	0
<i>Pseudomonas</i>	0	1.02	0.03	3.20	0.08	2.18	0.68	1.79	0.02	0	0.05	0	5.66
<i>Knoellia</i>	0.01	0	11.3	0	0.01	0	0	0	0	0	0	0	0
<i>Maricaulis</i>	0	0	0	0	0	0	0	10.7	0	0	0	0	0
<i>Geitlerinema</i>	1.78	0.11	0	5.76	0.02	0.31	0	0	0	0.03	0.02	2.34	0.34
<i>Microbacterium</i>	0	0	0	0	1.10	0	7.54	0	0	0.04	0	0	1.80
<i>Micrococcus</i>	0.03	0	0	17.3	0.13	0.31	7.87	0.03	0	0	0.77	0.82	0
<i>Ewingella</i>	0	0	0	0	0	0	0	2.03	9.29	0	0	0	0
<i>Bacillus</i>	0	0	0	2.32	0.07	5.23	3.07	0.06	0	0	7.13	7.35	0
<i>Roseomonas</i>	0.43	0	0	0	0.44	0	0	0	0	0	0	0	7.93
<i>Xylanimicrobium</i>	0.58	0.02	7.47	0	0	0	0	0	0	0	0	0	0
<i>Kineococcus</i>	1.65	0.02	7.18	0	0.07	0	0	0	0	0	0	0	0
<i>Thermomonas</i>	0	0	7.58	0	0	0	0	0	0	0	0	0	0
<i>Veillonella</i>	0.01	0	0	0	0	0	0	0	0	0	0.15	1.22	5.75
<i>Ralstonia</i>	0	0	0.86	0	0	0	0	1.26	5.53	0	0	0	0.60
<i>Solimonas</i>	0	0	0	0	0	6.90	0	0	0	0	0	0	0
<i>Comamonas</i>	0.03	0.04	0.07	0.03	0	1.37	0.02	2.03	0.29	0	0.98	0.15	1.72
<i>Streptomyces</i>	0	0	0	0.20	0.13	0	0.93	1.11	0.31	0.03	4.99	0	0
<i>Stenotrophomonas</i>	0	0	0	0	0	4.25	0	0	1.88	0.01	0.01	0.22	0.15
<i>Janibacter</i>	0	1.03	0	0	0.09	2.77	3.42	0	0	0	0	0	0
Other genera	3.51	3.43	4.84	1.85	2.05	3.01	3.92	3.33	6.84	0.36	2.09	5.86	1.05

Note: 19, 21, 22, 23, 24 are car identifiers and locations are A – steering wheel, C – gear shift knob, D – area near cup holder (see Figure 1A). The relative percentage of sequences assigned to a given taxonomic classification (genera) for each individual sample is arranged from highest to lowest across samples. The 36 most abundant genera are shown and the remaining that were at low levels are grouped as together as ‘other genera’.

3.2%, car 21: 4.1%, car 22: 8.5%, car 23: 38.7%, car 24: 7.5%); 9.47% were Betaproteobacteria (car 19: 3.3%, car 21: 12.7%, car 22: 5.6%, car 23: 8.2%, car 24: 17.8%); 6.8% were Alphaproteobacteria (car 19: 4.1%, car 21: 2.5%, car 22: 7.2%, car 23: 12.1%, car 24: 8.2%), and 5.6% were Clostridia (car 19: 1.9%, car 21: 8.9%, car 22: 3.1%, car 23: 5.3%, car 24: 9.1%). The remaining 7.5% comprised 10 other bacterial classes.

### **Staphylococcus species present in car interiors**

Because the genus *Staphylococcus* was determined to be a dominant member of the car interior microbiome and many staphylococcal species have the potential to be dangerous opportunistic pathogens, the identity of the staphylococcal species present was determined. Isolates that grew on nutrient agar and blood agar plates for the

culture-dependent CFU analysis (Figure 1B) were patched onto mannitol salt agar, which is a selective medium for members of the *Staphylococcus* genus. DNA was isolated from 87 patched colonies that grew on mannitol salt agar, 16s rRNA DNA was amplified and sequenced, and the resulting sequences were analyzed to determine *Staphylococcus* species (Table 2). *Staphylococcus epidermidis*, *S. aureus*, and *S. warneii* were found to be the most frequently isolated staphylococci, making up 87% of the staphylococcal isolates (representing 43, 31, and 13% respectively).

### **Antimicrobial susceptibility patterns of *S. aureus* isolates**

The ability to withstand antibiotic treatment is one of the reasons *S. aureus* is a prominent and dangerous

Table 2. Staphylococcal species present at different swab locations. Ratio indicates number of positive samples per *Staphylococcus* organism isolated at the site.

Swab location	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. caprae</i>	<i>S. sciuri</i>	<i>S. capitis</i>	<i>S. cohnii</i>	<i>S. saprophyticus</i>	<i>S. xylosus</i>	<i>S. warneii</i>	<i>S. hominis</i>	<i>S. haemolyticus</i>
A	8/18	9/18	0/18	0/18	0/18	0/18	0/18	0/18	1/18	0/18	0/18
B	2/9	5/9	1/9	0/9	0/9	0/9	0/9	0/9	1/9	0/9	0/9
C	6/15	6/15	0/15	0/15	0/15	0/15	0/15	0/15	1/15	1/15	1/15
D	5/18	10/18	0/18	0/18	0/18	0/18	0/18	0/18	3/18	0/18	0/18
E	2/9	4/9	1/9	0/9	0/9	0/9	0/9	0/9	1/9	0/9	1/9
J	2/10	3/10	0/10	0/10	1/10	0/10	0/10	0/10	4/10	0/10	0/10
K	2/8	1/8	0/8	1/8	1/8	1/8	1/8	1/8	0/8	0/8	0/8
A–K	27/87	38/87	2/87	1/87	2/87	1/87	1/87	1/87	11/87	1/87	2/87

Table 3. Antimicrobial susceptibility profiles for methicillin sensitive (MSSA) and methicillin resistant (MRSA) *S. aureus* isolates collected from shared cars.

Antimicrobial	Number of susceptible isolates (%) among	
	MSSA (n = 120)	MRSA (n = 35)
Penicillin	11 (9.1)	0 (0)
Oxacillin	120 (100)	0 (0)
Erythromycin	94 (78.3)	8 (22.8)
Gentamicin	120 (100)	35 (100)
Levofloxacin	120 (100)	12 (34.3)
Rifampin	120 (100)	35 (100)
Tetracycline	118 (98.3)	33 (94.3)
Vancomycin	120 (100)	35 (100)

pathogen. Of particular concern is methicillin-resistant *S. aureus* (MRSA), which has become a major nosocomial and community pathogen. *S. aureus* strains that are methicillin resistant have a *mecA* gene that encodes for a unique penicillin-binding protein that has decreased affinity for  $\beta$ -lactam antibiotics (Lambert 2005). To determine the prevalence of MRSA isolates from car interiors, the steering wheels of 30 community-shared cars (Zipcars) were swabbed and streaked onto mannitol salt agar. Colonies that grew and were able to ferment mannitol were confirmed to be *S. aureus* by 16s rRNA amplification and sequencing. On average, at least five *S. aureus* isolates (155 total) from each of the 30 cars was tested for methicillin resistance by plating culture dilutions onto tryptic soy agar containing  $10 \mu\text{g ml}^{-1}$  methicillin. Of the 155 isolates tested, 35 (22.6%) were found to be methicillin resistant and these isolates were confirmed as *mecA* positive by PCR. Isolates (35 MRSA and 120 MSSA) were also tested for susceptibility to other clinically relevant antibiotics (Table 3). All MSSA isolates were sensitive to oxacillin, gentamicin, levofloxacin, rifampin, and vancomycin. In addition most were sensitive to tetracycline (98%) and erythromycin (78%), but only 9% were sensitive to penicillin. The 35 MRSA

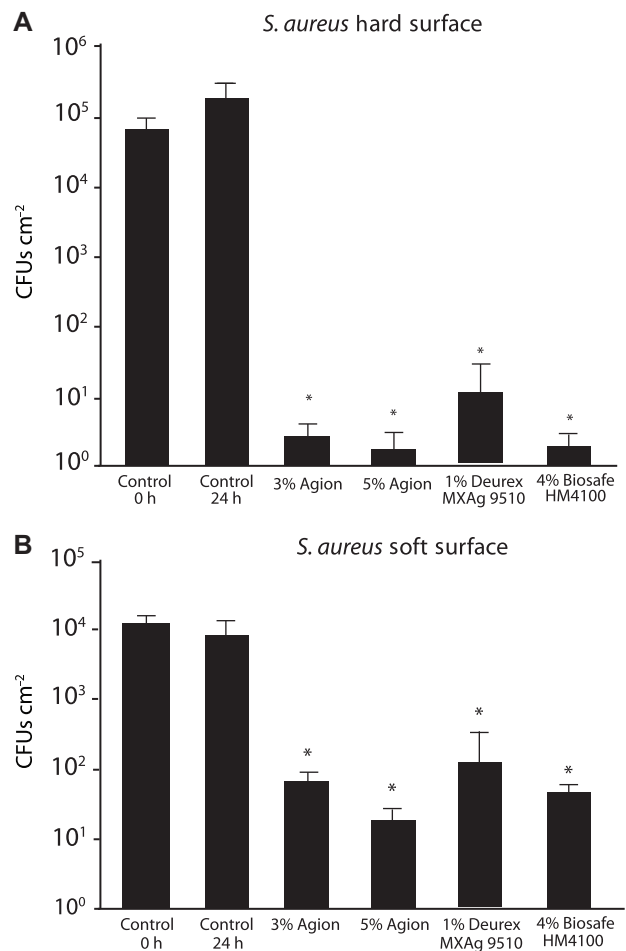


Figure 2. Analysis of car interior plastics treated with antimicrobial formulations for the ability to resist *S. aureus* surface colonization. Hard plastic surfaces (A) or soft plastic surfaces (B) that were either untreated (control) or sprayed with the indicated antimicrobial formulations were exposed to  $6 \times 10^5$  CFUs of *S. aureus* and the number of CFUs present after 24 h was determined. Error bars show the SE of the mean; \* $p < 0.005$  vs 24 h control.

isolates were all sensitive to gentamicin, rifampin, and vancomycin, and 94% were sensitive to tetracycline.

Susceptibility to levofloxacin and erythromycin was 34% and 22% respectively, and as expected all were resistant to the  $\beta$ -lactams penicillin and oxacillin.

**Analysis of antimicrobial surfaces for *S. aureus* colonization**

Considering that antibiotic resistant *S. aureus* was frequently found in car interiors and this environment might serve as a fomite that could lead to increased colonization of occupants, strategies to reduce or eliminate *S. aureus* colonization were investigated. Since most interior touch surfaces are painted to enhance the compo-

nent's appearance or feel, antimicrobial additives were added to the typical coating formulations to assess the efficacy in inhibiting microbial colonization (Figure 2). Both steering wheel grip (soft) and center trim (hard) coating formulations were assessed for the ability to resist *S. aureus* colonization. Four automotive paint formulations containing commercially available antimicrobial additives (3% silver ion, 5% silver ion, 1% nanosilver coated micronized wax, and 4% silane quaternary ammonium salt) were used to coat both hard and soft surfaces and all of these significantly reduced the ability of *S. aureus* to colonize the surface over a 24 h time period (Figure 2A and 2B). To test the durability of these antimicrobial coated surfaces, they were exposed to an artificial weathering process that mimics exposures

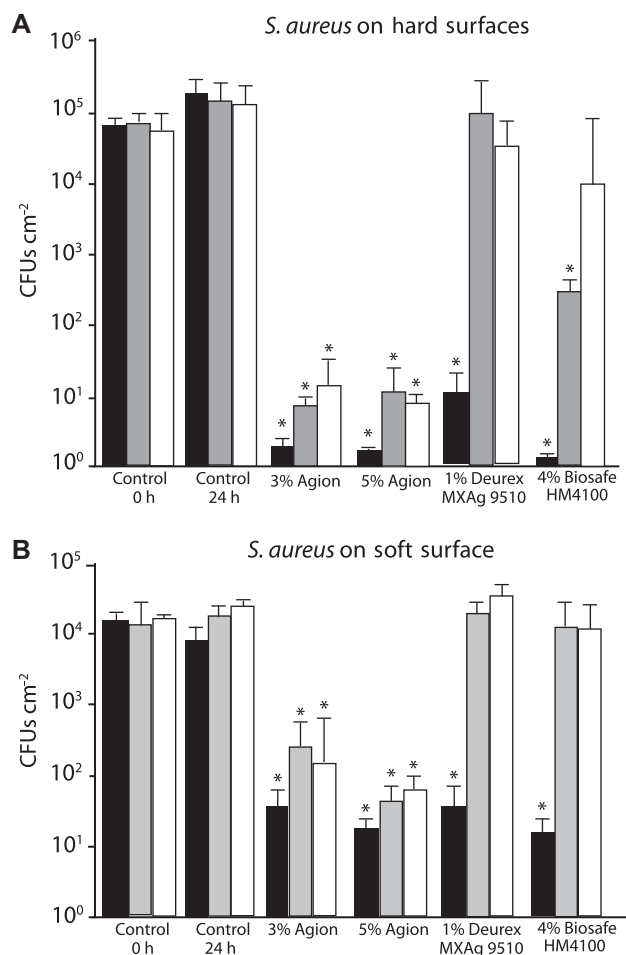


Figure 3. Analysis of artificially weathered car interior plastics treated with antimicrobial formulations for the ability to resist *S. aureus* surface colonization. Hard plastic surfaces (A) or soft plastic surfaces (B) that were either untreated (control) or sprayed with indicated antimicrobial formulations were artificially weathered then incubated with upto  $6 \times 10^5$  CFUs *S. aureus*. The number of CFUs present after 24 h was determined. Black bars are unweathered surfaces, gray bars were exposed to  $2,500 \text{ kJ m}^{-2}$ , and white bars were exposed to  $5,000 \text{ kJ m}^{-2}$ . Error bars the SE of the mean; \* $p < 0.005$  vs the comparable 24 h control.

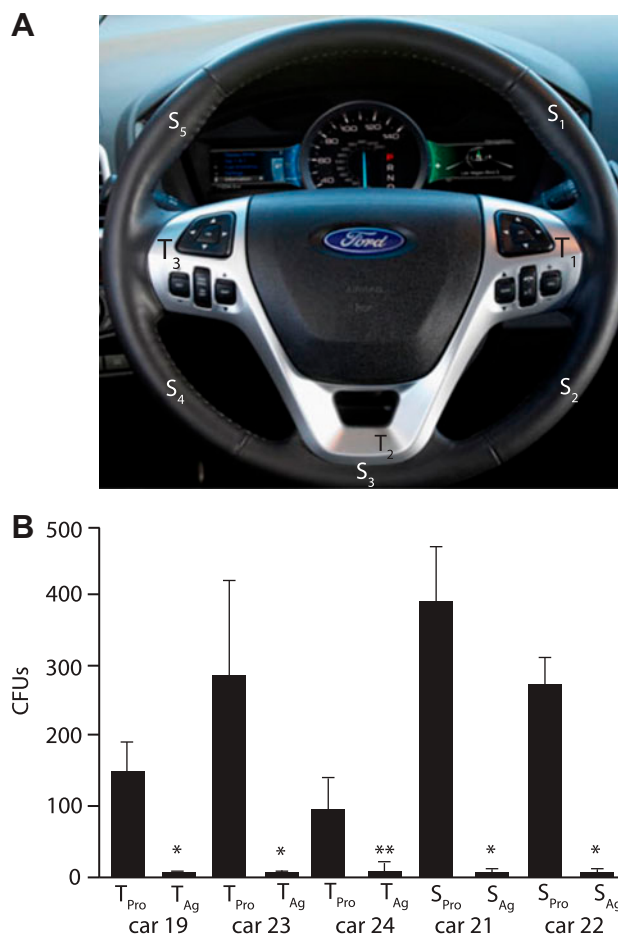


Figure 4. Analysis of CFUs present on production steering wheels vs silver treated steering wheels. (A) Locations of swabbing at hard trim piece locations (T1–3) or soft exterior locations (S1–5). (B) Number of CFUs isolated from swab locations from trim (T) or exterior steering wheel locations (S) from production (Pro) vs silver coated (Ag) steering wheels. \* $p < 0.005$  vs the comparable production piece; \*\* $p < 0.05$  vs the comparable production piece.

likely to occur inside an automobile (UV light, heat, and humidity conditions common to car interiors). The 1% nanosilver (Deurex) and 4% silane quaternary ammonium salt (Biosafe) treated surfaces lost anti-*S. aureus* colonization properties after artificial weathering, whereas the silver ion treated surfaces retained the ability to resist colonization (Figure 3).

#### **Analysis of antimicrobial surfaces in cars**

Next 5% Agion silver ion treated surfaces were examined to determine whether they would reduce pathogen colonization when placed in automobiles. The steering wheels of five cars from the Ford Motor Company motor fleet which are driven daily were swabbed at hard trim piece locations (T<sub>1-3</sub>) or soft exterior locations (S<sub>1-5</sub>) on standard production steering wheels (Figure 4A). Swabs of the non-silver treated production steering wheel locations were plated on nutrient agar and TSA blood agar to determine total CFUs per location (Figure 4B, T<sub>Pro</sub> & S<sub>Pro</sub>). Swabs were also plated onto mannitol agar and *S. aureus* and *S. epidermidis* were confirmed to be found on every production steering wheel by 16s rRNA analysis. After swabbing, the production steering wheel was removed and replaced with a 5% silver ion treated steering wheel. Five months later the steering wheel was swabbed at the same locations and CFUs were determined (Figure 4B, T<sub>Ag</sub> & S<sub>Ag</sub>). All swabs from the silver treated steering wheels had significantly fewer CFUs. 16s rRNA analysis of the colonies that came from swabbed silver treated steering wheels revealed all of the isolates were members of the *Bacillus* genus (*B. subtilis*, *B. sphaericus*, *B. firmus*, *B. megaterium*, *B. simplex*).

#### **Discussion**

Despite the fact that humans spend a significant amount of time inside automobiles, very little is known about the microbial ecology of car interiors and how this might impact the occupant's microbiome. In this study, bacterial communities of frequently touched car interior surfaces were enumerated and analyzed. These results indicate that the most highly colonized locations were areas that would be suspected to have frequent touching by the occupants, including locations on the steering wheel, the gear shifter, door handles and window switches, and the center console near the beverage holder (Figure 1). The bacterial communities of steering wheels, gear shifters, and the center console were analyzed by culture independent pyrosequencing and though this revealed novel populations at each site, two genera, viz. – *Staphylococcus* and *Propionibacterium* were the dominant members at most sites. Members of these

genera commonly colonize human skin, which suggests the prevalent genera which are able to persist on car interior surfaces are deposited there by skin to surface contact.

Because several members of the staphylococcal genus are important human pathogens, the species present were determined and it was found that the most prevalent were *S. epidermidis* (43%), *S. aureus* (31%), and *S. warneii* (13%) (Table 2). A relatively high percentage (22.6%) of methicillin resistant *S. aureus* was also isolated from shared community driven cars (Table 3).

This analysis suggests that car interiors may be important environmental reservoirs that are capable of harboring antibiotic resistant *S. aureus*. This is of potential interest because colonization by *S. aureus* significantly increases the likelihood of a person developing several types of infections (Stenehjem & Rimland 2013). *S. aureus* can readily be transferred from fomites to people. Colonized individuals can shed the organism into the environment, contaminating surfaces, which would allow for transfer to other individuals. *S. aureus* infection is notable for its ability to repeatedly infect patients, especially at sites involving the skin (Kaplan 2005; Crum et al. 2006) and there are indications that transmission among household members is common (Beguir et al. 2004; Miller & Diep 2008). Therefore, limiting skin exposures *via* fomites may be one useful strategy to reduce infection and spread in these scenarios.

In order to explore strategies that limit colonization by pathogenic microbes, plastics used in the production of car interiors were coated with antimicrobial silver formulations and tested for their ability to resist colonization by *S. aureus*. All silver treated surfaces displayed the ability to resist *S. aureus* colonization. However exposure to artificial weathering revealed that some treated surfaces (Agion) retained anti-colonization properties, while others lost the ability to resist colonization (Deurex MXAg9510 and Biosafe). It is unclear why weathering caused these differences.

Finally the ability of 5% Agion silver ion treated surfaces in the form of steering wheels and trim within steering wheels was examined for the ability to resist pathogen colonization when placed inside cars. Initial swabbing of non-antimicrobial production steering wheels revealed relatively high levels of bacteria colonization by CFU analysis and the presence of *S. aureus* and *S. epidermidis* on each steering wheel. These production steering wheels were replaced with steering wheels that had been treated with 5% Agion, and five months later swabbing revealed significantly fewer CFUs and the absence of all staphylococcal species. The only culturable microbes present on these surfaces were members of the spore-forming *Bacillus* genus.



Overall these results suggest bacteria present on frequently touched car interior surfaces likely originate from human skin. The deposit of some of these microbes on car interiors could have undesired consequences. For example, many skin microbes have the capacity to produce unpleasant odors (Dumas et al. 2009; Ara et al. 2013). Others, such as *S. aureus*, have the capacity to render more serious consequences and could potentially be a threat to human health. In addition, the presence of drug resistant *S. aureus* in cars used by multiple people could lead to the dissemination of this pathogen.

Environmental sources of *S. aureus* are clearly not isolated to cars. In hospitals, MRSA is frequently isolated from nurse workstations, bed spaces, pagers, and stethoscopes (Smith 1996; Singh et al. 2002; Hardy et al. 2006). Measures to control MRSA in these environments have likely contributed to limiting MRSA infections (Vriens et al. 2002; Meek 2004) and this success lends credence to the notion that environmental sources are an important component of MRSA pathogenesis. It is possible that strategies to reduce *S. aureus* car interior colonization, like those examined here, could reduce transmission, colonization and infection by this dangerous pathogen. It is proposed that individuals experiencing frequent *S. aureus* skin infections or those concerned about *S. aureus* carrier status consider the surfaces of car interiors as potential reservoirs for *S. aureus*.

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