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Characterization of microbial contamination in United States Air Force aviation fuel tanks

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Abstract Bacteria and fungi, isolated from United States Air Force (USAF) aviation fuel samples, were identified by gas chromatograph fatty acid methyl ester (GC-FAME) profiling and 16S or 18S rRNA gene sequencing. Thirty-six samples from 11 geographically separated USAF bases were collected. At each base, an above-ground storage tank, a refueling truck, and an aircraft wing tank were sampled at the lowest sample point, or sump, to investigate microbial diversity and dispersion within the fuel distribution chain. Twelve genera, including four *Bacillus* species and two *Staphylococcus* species, were isolated and identified. *Bacillus licheniformis*, the most prevalent organism isolated, was found at seven of the 11 bases. Of the organisms identified, *Bacillus* sp., *Micrococcus luteus, Sphinogmonas* sp., *Staphylococcus* sp., and the fungus *Aureobasidium pullulans* have previously been isolated from aviation fuel samples. The bacteria *Pantoea ananatis*, *Arthrobacter* sp., *Alcaligenes* sp., *Kocuria rhizophilia, Leucobacter komagatae, Dietza* sp., and the fungus *Discophaerina fagi* have not been previously reported in USAF aviation fuel. Only at two bases were the same organisms isolated from all three sample points in the fuel supply distribution chain. Isolation of previously undocumented organisms suggests either, changes in aviation fuel microbial community in response to changes in aviation fuel composition, additives and biocide use, or simply, improvements in isolation and identification techniques.

Keywords Aviation fuel · Microbial contamination · Fuel microorganisms · JP-8 · JP-4 · Jet-A

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Introduction

Microbial contamination in United States Air Force (USAF) aviation fuel was initially investigated in the 1950s [4, 7, 12, 21]. Microbial growth in aviation fuel storage tanks and aircraft wing tanks caused fuel filter plugging, corrosion, and increased maintenance costs associated with these problems[12, 13]. The importance of understanding the hazards associated with microbial contamination was underscored in 1958 when a B-52 crash was directly attributed to the plugging of an in-line fuel filter. Introduction of the fuel system icing inhibitor, ethylene glycol monomethyl ether (EGME) in 1962, coupled with improved ''housekeeping'', reduced the number of microorganisms in field fuel systems [2, 12]. Shortly thereafter, microbial research associated with USAF aviation fuel was abandoned. In 1984 the USAF replaced EGME with a more environmentally acceptable icing inhibitor, diethylene glycol monomethyl ether (Di-EGME) [3, 21, 23]. In addition, the transition in the 1980s from JP-4, a ''wide-cut'' fuel, to JP-8, a kerosene fuel, as well as the more recent addition of metal deactivating compounds, static dissipater agents, antioxidants and other chemical additives may have driven changes in the constitution of the microbial communities. Recent microbial contamination studies have been conducted on commercial aviation fuel; however, differences in the composition of commercial versus military aviation fuel may negate the validity of those results

Table 1 Microbial contaminants isolated from commercial and military aviation fuel from 1958 to the present

	JP-4 1958–1966 [4, 12, 13, 17]	Jet A 1988–1997 [27, 31]	Jet A-1 1998 & 1999 [11, 15]	JP-8 2002 [9]	JP-8 (current study)
Bacteria					
Acinetobacter (*calcoaceticus*, *cerificans*)		Yes	Yes		
Arthrobacter			Yes		Yes
Aerobacter aerogenes	Yes	Yes	Yes		
Aeromonas sp.		Yes	Yes		
Alcaligenes		Yes	Yes		Yes
Brevibacterium ammoniagenes	Yes		Yes		
Desulfovibrio sp. (SRB)	Yes	Yes	Yes		
Dietzia sp.					Yes
Escherichia sp.	Yes				
Enterobacter (*cloacae*, *glomerans*)			Yes		
Flavobacterium (*arborescens*, *diffusum*)	Yes	Yes	Yes		
Kocuria rhizophilia					Yes
Leucobacter komagatae					Yes
Micrococcus sp.	Yes	Yes	Yes		Yes
Pantoea ananatis					Yes
Streptomyces sp.			Yes		
Staphylococcus sp.					Yes
Sphingomonas					Yes
Serratia (*marcescens*, *odorifera*)			Yes		
Bacillus sp. (*acidocaldarius* + others)	Yes	Yes	Yes	Yes	Yes
Pseudomonas sp. (*aeruginosa* + others)	Yes	Yes	Yes		
Fungi					
Acremonium sp. (*strictum*)		Yes	Yes		
Aspergillus sp. (*niger*, *fumigatus* + others)	Yes	Yes	Yes		
Aureobasidium pullulans	Yes		Yes		Yes
Candida sp. (*famata*, *lipolytica* + others)		Yes	Yes		
Discophaerina fagi					Yes
Exophiala jeanselmei				Yes	
Fusarium sp. (*moniliforme* + others)		Yes	Yes		
Hormoconis (*Cladosporium*) *resinae*	Yes	Yes	Yes	Yes	
Helminthosporium sp.	Yes		Yes		
Paecilomyces (*variotii* + others)	Yes	Yes	Yes		
Penicillium sp. (*corylophilum* + others)	Yes	Yes	Yes		
Phialophora sp.		Yes	Yes		
Rhinocladiella sp.			Yes		
Rhodotorula sp.		Yes	Yes		
Trichosporium sp.			Yes		
Tothersrichoderma sp. (*viride* + others)		Yes	Yes		

for USAF fuel systems. Table 1 summarizes the results of several fuel contamination studies.

Since the diversity and severity of microbial growth within current USAF aviation fuel systems were unknown, a reevaluation of the impacts of the contamination on the fuel and fuel handling systems was prudent. Identification of microorganisms currently in USAF fuel should allow prediction of biodeterioration processes and potential detrimental consequences. This information can assist in understanding inoculation routes and metabolic pathways as well as suggest methods to reduce, prevent, or eliminate microbial proliferation. Additionally, the global war on terrorism (GWOT), including operations Enduring Freedom and Iraqi Freedom, gives rise to a heightened need for immediate identification of any biological contaminant. Knowledge of the baseline organisms present in USAF aviation fuel will allow for quick differentiation of microorganisms introduced maliciously and hence prevent a potentially catastrophic mission impact.

Microorganisms were isolated from USAF operational aviation fuel samples (JP-8, with standard additives) from geographically separate bases to probe the effects of location and climate. Samples were collected from at least three points in each base aviation fuel supply distribution chain to examine dispersion of microbial species from storage tank to refueling truck to aircraft wing tank.

Materials and methods

Sample collection

Sump samples were collected from 11 USAF Bases located within the continental United States. At least three 1 l samples were collected at each base, including samples from long-term above-ground storage tanks, refueling trucks, and aircraft wing tanks. Samples were collected from the lowest accessible point into sterile

glass bottles, then shipped overnight to the laboratory at Wright-Patterson AFB. Samples were plated within 24–72 h of collection.

If samples contained an aqueous phase, that phase was selected for testing. If samples contained no free water, the fuel was tested. One milliliter of sample was added to 9 ml sterile water and was then used for successive 1:10, 1:100 and 1:1,000 serial dilutions. One hundred microliters of neat sample, and each of the serial dilutions, were spread on each of three types of agar—trypticase soy broth (TSBA) (Becton Dickinson Microbiology Systems, Sparks, MD, USA), brain heart infusion with blood (BHIBLA) (REMEL, Lexena, KS, USA), and Sabouraud dextrose agar (SDA) (Becton Dickinson Microbiology Systems). TSBA and SDA plates were incubated at 28 °C and BHIBLA plates at 35 °C. BHIBLA plates were kept in an anaerobic environment with BBL GasPak Pouch Anaerobic Systems (Becton Dickinson Microbiology Systems). When obvious growth appeared on plates, colonies were restreaked onto new plates and incubated either 24 h (TSBA, SDA) or 48 h (BHIBLA). Media and growth conditions were selected for consistency with the GC FAME protocol and because they are known to support a wide range of microbial growth.

GC FAME

For GC-FAME analysis, fatty acid methyl esters, extracted from harvested cells, were analyzed using the Sherlock Microbial Identification System (Microbial ID, Inc, Newark, DE, USA) coupled with an Agilent Technologies, Inc. 5890 gas chromatograph with autosampler. Organisms were identified by comparing fatty acid methyl ester profiles with profiles of organisms in the Microbial ID, Inc (MIDI) libraries. Profile comparisons generated a similarity index, and a similarity index of 0.6 or greater is considered a good identification [1].

16S/18S rDNA

Harvested cells, suspended in 10 µl sterile water, were heated at 99 °C for 10 min to liberate cellular DNA. Two microliters of lysed cell suspension were added to PCR reaction mixture containing: 5 µl REDTaq 10X reaction buffer with $MgCl_2$ (Sigma-Aldrich Co, St Louis, MO, USA), 1 µl 10 mM deoxynucleotide mix (Sigma-Aldrich Co), 1 µl 100 µM forward primer, 1 µl 100 µM reverse primer, 39 µl ddH_2O and 1 µl REDTaq DNA polymerase (Sigma-Aldrich Co). Primer sequences and references are listed in Table 2. A Primus thermocycler (MWG-Biotech, High Point, NC, USA) was used for PCR. The PCR profile consisted of initial denaturation at 95 °C for 2 min, 45 °C for 1 min, 72 °C for 0.5 min followed by 25 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 30 s. PCR samples were analyzed by agarose gel electrophoresis. To verify amplification, bands were compared with 1 kB DNA ladder standard (Sigma-Aldrich Co). Once amplification was verified by electrophoresis, the PCR amplimers were cloned into a plasmid vector using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA) to amplify the fragments, according to manufacturer's protocol.

Table 2 Primer sequences used for PCR

Primer	Sequence (5′ to 3′)							
FuelbugF	TGG	AGA	GTT	TGA	TCC	TGG	CTC	A
FuelbugR	GCT	GCT	GGC	ACG	TAG	TTA	GC	
Fuelfun2F	CAA	AGA	TTA	AGC	CAT	GCA	TGT	
Fuelfun2R	AGA	CTT	GCC	CTC	CAA	TTG	TT	
A1369F[a]	CGG	TGA	ATA	YGY	CCC	TGC		
P1541R[b]	AAG	GAG	GTG	ATC	CRG	CCG	CA	
B1369F[a]	CGG	TGA	ATA	CGT	TCY	CGG		
P1492R[c]	GGW	TAC	CTT	GTT	ACG	ACT	T	
BSSAF[a]	ACG	ACG	GYG	GCA	TTT	CTC		
BSSAR[d]	GCA	TGA	TSG	GYA	CCG	ACA		

Primer sets	Specificity	Amplification size (bp)	Reference
FuelbugF/FuelbugR	Bacteria 16S rRNA gene	500	[8, 9, 33]
Fuelfun2F/Fuelfun2R	Fungal 18S rRNA gene	500	[20]
A1369F/P1541R	Archea 16S rRNA gene	172	[30]
B1369F/P1492R	Bacteria 16S rRNA gene	123	[30]
BSSAF/BSSAR	Archea catabolic gene for toluene, xylene degradation	132	[5]

Degenerative primers were synthesized where nucleotides are annotated with Y, S, W or R
[a] Y = C or T
[b] R = A or G
[c] W = A or T
[d] Y = C or T, S = C or G

Viable, white colonies were picked and grown aerobically overnight at 37 °C in sterile Luria-Bertani (LB) broth, supplemented with 100 μg/ml ampicillin for plasmid selection. Plasmid DNA purification was accomplished using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Purified DNA was digested with *Eco*RI restriction enzyme (Roche Biochemicals, Indianapolis, IN, USA) and the digested products were separated by agarose gel electrophoresis to confirm presence of the DNA insert. Plasmid DNA containing the PCR fragments was subjected to cycle sequencing reactions using Big Dye version 2 dye terminator reaction mix (Applied Biosystems, Foster City, CA, USA) and M13 reverse and T7 promoter primers (Invitrogen and IDT, Coralville, IA, respectively) in a Perkin-Elmer 2700 thermocycler (Perkin-Elmer, Foster City, CA, USA). Completed reactions were purified using isopropanol (Amresco, Solon, OH, USA) precipitation prior to denaturation and analysis with an ABI 3100 genetic analyzer (Applied Biosystems). Resulting sequences were compared with the National Center for Biotechnology Information (NCBI) public domain site to identify organisms (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Thirty-six samples from 11 USAF bases, shown in Table 3, were collected because of reports that water was routinely collected from tank sumps. Water in sump samples is a strong predictor of microbial growth [11]. Additionally, these bases were geographically separated, located in the Northern, Southern, Eastern and Western regions of the continental United States.

Sample appearance varied greatly from base to base. Some were clean, clear and contained only trace amounts of water. Others were dark in color, ranging from yellow to brown, with obvious solids and significant amounts of water. Water pH ranged from 5.4 – 7.2.

Twenty-eight of the 36 samples contained culturable microbial contaminants. Of the organisms cultured, 24 came from fuel samples and 35 from water samples. Populations recovered ranged from 10^2 to 10^6 CFU/ml. Media and growth conditions were clearly selective for certain organisms. The BHI medium and anaerobic conditions accounted for nearly all the *B. licheniformis* isolates. The two fungal isolates were from SDA plates.

GC-FAME and DNA sequencing results are compared in Table 3. The DNA sequencing identified ten bacterial genera, *Alcaligenes, Arthrobacter, Bacillus, Dietzia, Kocuria, Leucobacter, Micrococcus, Pantoea, Sphingomonas, Staphylococcus*, and two closely-related fungal genera, *Aureobasidium* and *Discophaerina*. The most commonly isolated organism, *Bacillus licheniformis*, was identified from seven of the 11 bases. The GC-FAME results were similar, but not identical. Nine bacterial genera, with a total of 17 species were identified. Genera identified by GC-FAME (and their similarity index), but not by DNA analyses, were *Actinomadura* (0.018), *Brevibacterium* (0.157), *Brevundimonas* (0.016 to 0.144), *Cellulomonas* (0.368), *Rhodococcus* (0.437), and *Xenorhabdus* (0.583 to 0.675). Of these, only *Xenorhabdus* met the criteria for a confident identification, a similarity index $=0.6$. Seven of the 28 samples gave precisely the same result by both identification techniques: *Bacillus megaterium*, *B. cereus*, two *B. licheniformis* cultures, two *Micrococcus luteus* cultures, and *Staphylococcus warneri*.

Only at Hurlburt AFB, FL, Ellsworth AFB, SD and Holloman AFB, NM were the same organisms found in all three of the samples taken. At Hurlburt and Ellsworth, *Bacillus licheniformis*, and at Holloman, *Sphinogmonas* sp. was isolated from storage tanks, refueling trucks, and aircraft wing tanks.

Thirteen of the 16 bacterial species isolated and identified by DNA analyses were Gram-positive. Both aerobic and facultative anaerobic organisms were found. Most were common soil or airborne organisms [14]. Five were actinomycetes.

The nine genera and 17 species of bacteria identified by GC-FAME were of similar taxonomic groups as those identified by DNA analyses. The two Gram negative genera, *Brevundimonas* and *Xenorhabdus* are of the same class as two of the Gram-negatives from DNA analyses, *Alpha* and *Gamma* Proteobacteria, *Sphingomonas* and *Pantoea*, respectively. Likewise, the different Gram positive organism identifications, *Paenibacillus* and *Cellulomonas*, are of the same Sub-Class, the *Actinobacteridae*, as the DNA-based identifications [14].

The two fungi, *Aureobasidium pullulans* and *Discophaerina fagi* appear closely related, and are common soil organisms [10, 29].

Discussion

Microorganisms previously isolated from aviation fuel are compared with results from this investigation in Table 1, including the military aviation fuels JP-4 and JP-8, as well as the commercial aviation fuels Jet-A and Jet-A1. *Bacillus* sp., *Micrococcus* sp., *Sphingomonas* sp., *Staphylococcus* sp. and *Aureobasidium pullulans* have all been found previously in USAF aviation fuel and were confirmed as still viable contaminates by this study [7, 12, 22]. *Pantoea ananatis, Arthrobacter* sp., *Alcaligenes* sp, *Kocuria rhizophilia*, *Leucobacter komagatae* and *Dietzia* sp. have not been previously isolated and identified from USAF aviation fuel. However, *Arthrobacter* sp., and *Alcaligenes* sp. were recently identified in commercial aviation fuel [11, 15]. *P. ananatis, K. rhizophilia, L. komagatae* and *Dietzia* sp. have not been documented as previously isolated from either commercial or USAF aviation fuel.

While the isolation of *Kocuria* sp. and *Dietzia* sp. from aviation fuel has not been previously noted, both *Kocuria* sp. and *Dietzia* sp. have been isolated from the waters of an oil field and shown to utilize hydrocarbons

Table 3 Sample locations and organism identifications

Collection site (USAF Base)	Source	Phase (fuel) (water)	Media	16Sr (18Sr*)	Percent ID	GC-FAME	Similarity index
Davis-	Aircraft	F	TSB	*Arthrobacter* sp.	99	No match	–
Monthan, AZ	Truck	W	TSB	*Leucobacter komagatae*	96	No match	–
Edwards, CA	Tank	W	TSB	*Pantoea ananatis*	99	*Xenorhabdus nematophilus*	0.588
	"	W	SDA	*Aureobasidium pullulans**	98	No match	–
	Truck 1	W	TSB	*Pantoea ananatis*	99	*Xenorhabdus nematophilus*	0.583
	Truck 2	W	TSB	*Pantoea ananatis*	98	*Xenorhabdus nematophilus*	0.675
Eglin, FL	Aircraft	F	BHI	*Sphingomonas* sp.	99	*Bacillus lentimorbus*	0.324
	Tank	F	BHI	*Bacillus licheniformis*	98	*Bacillus lentimorbus*	0.022
	"	F	TSB	*Kocuria rhizophilia*	94	*Micrococcus luteus*	0.654
	Truck	W	BHI	N/A	–	*Bacillus megaterium*	0.025
	"	W	TSB	N/A	–	*Actinomadura yumaensis*	0.018
Ellsworth, SD	Aircraft	F	BHI	*Bacillus licheniformis*	99	No match	–
	"	F	BHI	*Pantoea ananatis*	99	No match	–
	Tank	W	TSB	*Bacillus* sp.	99	*Bacillus cereus*	0.736
	"	W	TSB	N/A	–	*Bacillus licheniformis*	0.685
	"	W	TSB	*Micrococcus luteus*	100	*Micrococcus luteus*	0.426
	Truck	F	BHI	*Bacillus licheniformis*	100	*Bacillus licheniformis*	0.557
Hill, UT	Tank	F	TSB	*Bacillus pumilus*	99	No match	–
Holloman, NM	Aircraft	F	TSB	*Sphingomonas* sp.	99	*Brevundimonas vesicularis*	0.027
	"	F	TSB	N/A	–	*Cellulomonas flavigena*	0.368
	"	F	SDA	N/A	–	*Bacillus licheniformis*	0.522
	"	F	SDA	*Discophaerina fagi**	95	*Bacillus licheniformis*	0.360
	"	F	BHI	*Staphylococcus epidermidis*	98	No match	–
	"	F	BHI	*Bacillus* sp.	98	No match	–
	Tank	F	TSB	N/A	–	*Bacillus licheniformis*	0.734
	"	F	BHI	*Bacillus licheniformis*	99	No match	–
	"	F	SDA	N/A	–	*Rhodococcus bronchialis*	0.437
	"	F	TSB	*Sphingomonas* sp.	97	*Brevundimonas vesicularis*	0.016
	Truck	W	TSB	N/A	–	*Brevundimonas vesicularis*	0.144
	"	W	TSB	*Sphingomonas* sp.	92	No match	–
	"	W	TSB	N/A	–	*Brevibacterium epidermidis*	0.157
	"	W	SDA	N/A	–	*Bacillus licheniformis*	0.517
Hurlburt, FL	Aircraft	W	TSB	*Staphylococcus warneri*	98	*Staphylococcus warneri*	0.248
	"	W	BHI	*Bacillus licheniformis*	96	*Bacillus lentimorbus*	0.014
	"	W	BHI	*Dietzia* sp.	98	No match	–
	"	W	BHI	*Staphylococcus warneri*	97	No match	–
	"	W	TSB	*Staphylococcus epidermidis*	98	*Staphylococcus cohnii cohnii*	0.325
	Tank	F	BHI	*Bacillus licheniformis*	98	No match	–
	"	F	TSB	*Staphylococcus epidermidis*	97	*Staphylococcus warneri*	0.238
	"	F	TSB	N/A	–	*Staphylococcus cohnii cohnii*	0.299
	Truck	W	BHI	*Bacillus licheniformis*	99	No match	–
	"	W	TSB	N/A	–	*Bacillus pasteurii*	0.780
	"	W	TSB	N/A	–	*Bacillus megaterium*	0.478
Kirtland, NM	Truck	W	BHI	*Bacillus subtilis*	99	No match	–
	"	W	TSB	N/A	–	*Bacillus subtilis*	0.772
Moody, GA	Tank	W	SDA	*Bacillus pumilus*	98	No match	–
	"	W	TSB	*Alcaligenes* sp.	99	*Bacillus pumilus*	0.329
	"	W	BHI	N/A	–	*Bacillus licheniformis*	0.526
	Aircraft	F	TSB	*Bacillus megaterium*	99	*Bacillus megaterium*	0.924
Tyndall, FL	Aircraft	F	BHI	N/A	–	*Bacillus licheniformis*	0.604
	Tank	F	BHI	*Micrococcus luteus*	100	*Micrococcus luteus*	0.212
	Truck	W	TSB	N/A	–	*Staphylococcus aureus*	0.508
Wright-Patterson, OH	Tank 1	W	BHI	*Bacillus pumilus*	98	*Bacillus licheniformis*	0.695
	"	W	BHI	*Bacillus licheniformis*	99	No match	–
	Tank 2	W	BHI	*Bacillus licheniformis*	99	No match	–
	Tank 3	W	TSB	*Bacillus cereus*	99	*Bacillus cereus*	0.743
	"	W	TSB	*Bacillus licheniformis*	97	*Bacillus licheniformis*	0.897
	"	W	BHI	*Bacillus licheniformis*	99	No match	–
	Tank 4	W	TSB	N/A		*Bacillus cereus*	0.567

Rows represent identification of a single isolate using both 16Sr (18Sr*) and GC-FAME techniques. If no identification is listed in column, no identification was conclusive for that isolate using that technique. N/A indicates that the DNA amplification was unsuccessful for that sample. No match indicates that the GC-FAME analysis was accomplished on colonies grown from the sample, however, no match was identified using the GC-FAME technique

[24]. Therefore, the adaptation of *Kocuria* sp. and *Dietzia* sp. to the aviation fuel environment is not surprising.

Pantoea ananatis, the bacterium responsible for onion center rot [16], has not been previously documented as associated with hydrocarbons or hydrocarbon degradation. *P. ananatis* was isolated from the refueling truck and two storage tanks at Edwards AFB, CA. The pipeline that supplies fuel to Edwards AFB passes through the San Joaquin Valley, one the of the main production areas for bulb onions. This circumstantial evidence, suggesting a possible pathway for *P. ananatis* contamination, underscores the complexity of identifying and eliminating all routes of inoculation of aviation fuel. These same cultures, when analyzed by GC-FAME, gave *Xenorhabdus nematophilus*, a member of the same bacterial family, the *Enterobacteriales*.

No information regarding *L. komagatae* is available other than the initial 1996 article describing the organism as an aerobic, gram-positive, non-sporulating rod. No instances of isolation from aviation fuel or other hydrocarbon source have been documented [32].

In addition to identifying organisms that have not been previously noted in aviation fuel, the verification of the contamination of USAF aviation fuel with known fuel contaminants gives insight into the current condition of USAF aviation fuel.

Bacillus sp. was found in the initial USAF aviation fuel microbial studies however, *B. licheniformis* was not specifically identified. In this study, *B licheniformis* was the most commonly isolated organism. *B. licheniformis* has been shown to actively produce biosurfactants which exhibit an oil-releasing effect [25]. The exceptional ability of this bacterium to liberate hydrocarbons from the fuel phase into the aqueous phase for metabolism may account for its proliferation throughout the USAF aviation fuel supply.

This study is the first instance of *Arthrobacter* sp. being isolated from USAF aviation fuel. The metabolites of *Arthrobacter* hydrocarbon oxidation have been shown to support sulfate-reducing bacteria (SRB) [19]. SRB microbial induced corrosion (MIC) of fuel tank walls is one of the biggest concerns regarding microbial contamination of aviation fuels [6, 26, 27]. Therefore, the isolation of *Arthrobacter* sp. from USAF aviation fuel maybe an indicator of the potential for SRB growth within storage tanks. If verified, exploitation of this relationship may facilitate early detection of environments conducive to SRB MIC thereby allowing for proactive remediation and prevention of soured fuel and/or corroded tanks.

This study investigated current microbial contamination in USAF aviation fuel and explored the possible transfer of organisms from one point in the fuel distribution line to another. Unlike commercial aircraft, which are loaded with only the fuel necessary for a mission, USAF aircraft often require defueling upon landing. Aviation fuel removed from aircraft wing tanks is returned to bulk storage. Samples were collected from an above-ground storage tank, a refueling truck, and an aircraft wing tank at each base in order to compare the microorganisms isolated from the distribution pathway. Only at three bases were the same microorganisms found in all three of the samples. Additionally, at only one base was the same organism found in two of the three samples. These data indicate that the contamination of the different distribution points is more complex than simple inoculation from points upstream. Vents are possible routes of exposure to environmental microorganisms for all three tank types. The practice of defueling and mid-air refueling aircraft greatly complicates unraveling the inoculation mechanism since military aircraft may take on fuel at one location, be refueled in route and defuel at yet another location.

Another purpose of this research was to evaluate the effects of sample collection location and climate on the diversity and levels of contamination. JMP Release 5.0 (JMP, Cary, NC, USA) statistical software was used in an attempt to correlate collection site environmental conditions (temperature, relative humidity, and average rainfall in last 30 days) to the quantities of microorganisms (data not shown). No statistically relevant information was gained from the correlation analysis. All samples for this study were collected between Sept–Nov 2002. Water contamination is believed to be the keystone for microbial proliferation [11]; therefore, further investigations of possible effects on microbial diversity and concentration by location and/or climate require a more extensive sampling protocol spanning, at minimum, the locales' dry and wet seasons.

Even though statistical analysis was not able to show dependence of sample quality on collection location, sample variability between sites was evident upon visual inspection. While a one liter sample was collected from each tank at each location the composition of that 1 l varied greatly. Most aircraft wingtank samples contained mostly fuel with a negligible aqueous phase while many storage tank samples were composed primarily of water. Therefore it was determined that the most applicable method for culturing viable organisms from all samples would be to use a modified solid media plating technique (ASTM D6469) and apply a 100 μL aliquot of either the aqueous phase, if present, or of the fuel phase to the appropriate media. Alternate methods discussed in both ASTM 6469 and IP 385/99 include filtering a constant sample volume and then placing the actual filter paper on the plate however, the variable sample compositions collected in this study made these methods impractical [28].

One of the concerns regarding identification of unknown microorganisms is inconsistent identification of the organisms by different laboratory techniques. These inconsistencies inevitably arise as each technique evaluates a specific characteristic of an organism which may be influenced by a variety of variables. In this study, the unknown microorganisms were identified using both

GC-FAME and 16S or 18S ribosomal RNA gene sequencing. While DNA sequencing gives rise to identifications based on organism-specific DNA sequence, the identifications resulting from GC-FAME are dependent on the organisms' environment. Temperature, growth media and cell age all influence cellular fatty acid composition. Even with stringent adherence to manufacturer protocols, there maybe slight differences between the cellular chemical composition of the unknown organism and the known organism strains used to create the commercial database. These differences may be exacerbated if an insufficient number of strains have been used to create the database [18]. A possible example of this occurring in this study was the GC-FAME identification of an aerobic organism isolated from the Wright-Patterson AFB storage tank as *Bacillus licheniformis* whereas DNA sequencing identified the organism as *Bacillus pumilus*, a phylogeneticly closely-related organism. Additionally, four of the 12 organisms identified through DNA sequencing in this study *Leucobacter komagatae, Kocuria rhizophilia, Aureobasidium pullulans* and *Discophaerina fagi* were organisms not included in the commercially available GC-FAME library and therefore could not be identified using that technique. Likewise, several organisms were not identified beyond genus by DNA analyses. This too represents limitations of taxonomic libraries based on genomics. As commercial libraries are updated, or as in-house libraries are created for unique applications, corresponding identifications between varying techniques should increase.

This study indicates that composition of microbial contamination of USAF aviation fuel has changed since the initial studies in the 1950s. Microbial contaminants identified included many organisms previously isolated from USAF aviation fuel as well as five new bacterial genera. The changes in the microbial community constitution may demonstrate microbial adaptation to changes in aviation fuel composition, additives and biocide use, or merely the result of improvements of isolation and identification techniques. While the causes of these compositional changes are of scientific interest and unraveling their derivation should be pursued, a major goal of this research was to document organisms indigenous to current USAF aviation fuel. None of the organisms identified are thought to be unique to the fuel environment; nevertheless, an understanding the normal bacterial and fungal flora of fuel systems will allow for quick determination of whether the presence of an organism and its impact on the aviation fuel system is unusual and should be of concern. Future research will include continuation of the monitoring the diversity of microorganisms in aviation fuel systems as well as investigation of the metabolism of USAF aviation fuel by the isolated organisms with the end objective of understanding detrimental effects and means of inhibition. Additionally, research efforts will continue to examine direct-PCR and other DNA-based field portable methods of detection.

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