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Bacterial Species Diversity in Cigarettes Linked to an Investigation of Severe Pneumonitis in U.S. Military Personnel Deployed in Operation Iraqi Freedom

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Abstract. This report presents results from a study on the bacterial diversity of cigarette brands collected from military personnel during the U.S. Army's investigation of a series of cases of acute eosinophilic pneumonitis in military personnel deployed in Operation Iraqi Freedom. Eight species of *Bacillus*, including five new species, and one new species of *Kurthia* were isolated from the cigarettes. Some of these species have been identified elsewhere as causes of hypersensitivity pneumonitis and other respiratory syndromes. All of the isolates were facultative anaerobes, and many displayed mucoid growth under anaerobic conditions. In addition, many isolates also displayed the ability to form surface biofilms under liquid culture. Although biofilm formation and mucoid growth were not correlated, the former was found to be much more pronounced under anaerobic conditions as opposed to aerobic ones. The implications of these findings are discussed.

Between March and August of 2003, approximately 19 United States military personnel deployed in Southwest Asia during Operation Iraqi Freedom developed severe pneumonitis from unknown causes [8, 33]. Early findings from the U.S. Army epidemiologic investigation team suggested that a large portion of these cases were consistent with a diagnosis of acute eosinophilic pneumonitis (AEP), which is an acute febrile illness without an identifiable infectious cause that may be at least partially caused by exposure to one or more allergens or other environmental agents [1]. The results of a one-year study conducted by the Army into the causes behind these cases of AEP concluded that there was an increased incidence of this disease among deployed military personnel in Southwest Asia [33], but they could not identify the specific causes underlying these cases of AEP. Nevertheless, the study revealed that all patients were tobacco users and that 78% began smoking tobacco just after deployment to the region. This link to tobacco smoking is important because previous research suggests a possible role for IgE-mediated hypersensitivity to cigarette smoke in pneumonia patients [23, 31, 32, 35, 39].

Previous studies on the bacterial load of cigarettes and other tobacco products show that they harbor potentially virulent organisms. For example, Eaton et al. [11] found *Mycobacterium avium* in cigarettes. This species is known to cause infections in persons with chronic lung disease [40], and it is a frequent source of infection for persons with AIDS [25]. Recently, Rubinstein and Pedersen [29] found that *Bacillus* isolates from chewing tobacco were capable of producing potent exogenous virulence factors that cause oral tissue damage. In fact, several studies have shown that *Bacillus* species can cause various types of pulmonary infections and inflammations, such as hypersensitivity pneumonitis and extrinsic allergic alveolitis caused by *B. subtilis* [12, 16, 37], a respiratory syndrome known as Machine Operator's Lung caused by *B. pumilus* [4], fulminating bacteremia and pneumonia caused by *B. cereus* [3, 22], and pseudomembranous tracheobronchitis caused by

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B. cereus [34]. In light of the various pulmonary diseases and allergic reactions caused by *Bacillus* species that have also been isolated from tobacco products and the observed high frequency of cigarette smokers among soldiers who contracted severe pneumonitis during Operation Iraqi Freedom, we conducted a study on the bacterial species diversity of cigarette brands available to U.S. servicemen in Southwest Asia that were collected by the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM).

Materials and Methods

Cigarette sampling, bacterial load enumeration, and strain isolation. Cigarettes actually used or carried by the case patients immediately prior to symptom onset were unavailable to the U.S. Army investigation team. Therefore, regionally representative cigarette samples were collected by U.S. Army preventive medicine assets from both local merchants and the U.S. military exchange in Baghdad, Iraq. Cigarette control samples were collected from the exchange at Aberdeen Proving Ground, Maryland. Samples were sent from the field to the USACHPPM at Aberdeen Proving Ground, Maryland, for distribution to supporting analytical laboratories [33]. Cigarette samples from the field consisted of two cartons per brand wrapped in cellophane inside a large cardboard box. At USACHPPM, samples were split and a portion of all samples collected were distributed to the U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS), and the National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois.

Researchers at NCAUR received 17 packs of cigarettes from the USACHPPM and stored at 4°C until processing (Table 1). Those that were collected from local merchants in Iraq and the U.S. military exchange in Baghdad were packaged in a variety of countries in Southwest Asia and elsewhere including France, Germany, Iraq, Jordan, South Korea, the United Kingdom, the United States, and Yemen. In order to facilitate statistical comparisons, we pooled the cigarette brands into three sample classes: American, European, and Southwest Asian. The sole brand from South Korea was treated separately.

The most probable number (MPN) method was used to enumerate the total bacterial load in sampled cigarettes according to procedures outlined by the U.S. Food and Drug Administration [5]. For each MPN enumeration, the filter was removed from the sampled cigarette in order to maximize the amount of tobacco included in the enumeration, as we anticipated this portion of the cigarette would contain most of the microbial population and that any microorganisms present in the filter would more than likely have come from the tobacco anyway [11]. Subsequently, 0.65 g of the remaining tobacco and paper was diluted in 64.5 ml of sterile distilled water with 0.1% peptone. The sample was then processed in a stomacher for 1 min in order to obtain complete homogenization. A series of 1:10 dilutions were made until the original was diluted to 1:10,000,000. Subsequently, 1 ml of each dilution was added to 9 ml of tryptone-glucose-yeast extract (TGY) broth and incubated aerobically at 28°C for 48 h. This procedure was repeated in triplicate for each cigarette brand.

Individual isolates were obtained from 10^{-2} MPN dilutions by streaking onto TGY agar plates and incubating aerobically at 28°C. The ability of each of these isolates to grow anaerobically was investigated by streaking onto CDC anaerobe blood agar with phenylethyl alcohol (herein referred to as "blood agar") (Becton Dickinson and Company, Sparks, MD, USA) and incubating at 28°C in a GasPak® Jar (Becton Dickinson and Company, Sparks, MD, USA) with a CO_2 + H_2 atmosphere. The blood agar used is selective for gram-positive isolates, but because we obtained no gram-negative isolates using nonselective TGY media, the use of blood agar to test for anaerobic growth is appropriate. In addition, the ability of each isolate to form a surface biofilm was investigated by incubating at 37°C both aerobically and anaerobically in 5 ml of Difco™ liver infusion (DL) broth (Becton Dickinson and Company, Sparks, MD, USA) without shaking for 48 h. Anaerobic biofilm incubations were conducted in a GasPak® Jar for 48 h (Becton Dickinson and Company, Sparks, MD, USA) with a CO_2 + H_2 atmosphere. The formation of a biofilm was confirmed if a pellicle was present at the liquid surface.

Strain identification. A fragment of the 16S rRNA gene corresponding to positions 518 to 1513 of the *E. coli* 16S rRNA was amplified from each isolate using the universal primers and polymerase chain reaction (PCR) protocol of Lu et al. [21]. The forward and reverse strands of each fragment were sequenced using the Big Dye® Kit (Applied Biosystems, Inc., Foster City, CA) on an ABI 3730 automated sequencer. After the resulting DNA sequences were verified, the online NCBI BLAST computer program was used to identify the closest 16S matches in GenBank. An alignment of these sequences was constructed by using the program ClustalX [36] and subsequently checked for errors by visual inspection. Each isolate was identified to species level through phylogenetic analyses, which were conducted using the neighbor-joining method [30] and Kimura [18] two-parameter distances with 1,500 bootstrap replicates in order to gauge the statistical reliability of internal branches. Because the 16S gene is not informative for discriminating between *B. subtilis* and certain closely related species [9, 24], an approximately 1-kb fragment of the *gyrA* gene was amplified using the primers and PCR protocol of Chun and Bae [9] in order to conduct species identification for isolates typed as *B. subtilis* on the basis of their 16S sequence identities. The methods of sequencing, alignment, and phylogenetic analysis of these sequences were the same as described above for the 16S rRNA gene sequences.

The 16S rRNA nucleotide sequences have been deposited in GenBank under the accession numbers AY663657–AY663689. The *gyrA* nucleotide sequences have been deposited in GenBank under the accession numbers AY663690–AY663711.

Results

Species identification. A total of 38 isolates were obtained and accessioned into the USDA ARS Culture Collection (http://nrrl.ncaur.usda.gov/). The isolates and their accession numbers are listed in Table 1. All of the isolates were identified as members of the Bacillaceae on the basis of 16S gene identities and gene clustering patterns (Fig. 1). Of these isolates, 4 were identified as *B. pumilus*, 4 were identified as *B. cereus*, and 22 were identified as members of the *B. subtilis* species group. Three new species were also found on the basis of the 16S phylogeny (Fig. 1). One species encompasses 6 strains related to *B. megaterium* (Fig. 1). Another strain, NRRL B-23979, is closely related to *Bacillus* KL-152, a strain recovered from the NASA Jet Propulsion Laboratory facilities [38] (Fig. 1). These strains appear to represent a new species that is very divergent from the

Table 1. Diversity of species recovered from cigarettes available to U.S. soldiers during Operation Iraqi Freedom[a]

Geographic origin[b]	Strain Number	Species	Colony Type[c]	Pellicle morphology[d]	
				Aerobic	Anaerobic
Europe	NRRL B-23981	*B. pumilus*	Dry	Thin	Smooth
Europe	NRRL B-23965	*B. cereus*	Dry	—	Very thin, ring
Europe	NRRL B-23966	*B. cereus*	Dry	—	—
Europe	NRRL B-41008	*B. subtilis*	Mucoid	Thin	Thin
Europe	NRRL B-23982	*B.* sp. 3	Dry	Incomplete	Incomplete
Europe	NRRL B-23940	*B.* sp. 4	Mucoid	—	—
Europe	NRRL B-23974	*B. subtilis*	Mucoid	Smooth	Smooth
Europe	NRRL B-23978	*B. subtilis*	Dry	Textured	Textured
Europe	NRRL B-23935	*B.* sp. 2	Mucoid	Textured	Textured
Europe	NRRL B-23979	*B.* sp. 5	Dry	—	—
Southwest Asia	NRRL B-23955	*B. cereus*	Dry	—	—
Southwest Asia	NRRL B-23957	*B. cereus*	Dry	—	Very thin ring
Southwest Asia	NRRL B-23934	*B. subtilis*	Dry	Thin	Thin
Southwest Asia	NRRL B-23973	*B.* sp. 1	Mucoid	Smooth	—
Southwest Asia	NRRL B-23956	*B.* sp. 3	Dry	Thin	Thin
Southwest Asia	NRRL B-41007	*B.* sp. 4	Mucoid	Cobweb	Cobweb
Southwest Asia	NRRL B-23954	*B.* sp. 1	Mucoid	Textured	Textured
Southwest Asia	NRRL B-23967	*B. subtilis*	Dry	Incomplete	Thin
Southwest Asia	NRRL B-23968	*B. subtilis*	Dry	Smooth	Thin
Southwest Asia	NRRL B-23972	*B. subtilis*	Dry	Thin	Thin
Southwest Asia	NRRL B-23936	*B.* sp. 1	Mucoid	Thin	Thin
Southwest Asia	NRRL B-23964	*B.* sp. 1	Mucoid	Thin	Smooth
Southwest Asia	NRRL B-41006	*B.* sp, 4	Mucoid	—	—
Southwest Asia	NRRL B-23939	*B.* sp. 4	Dry	—	—
Southwest Asia	NRRL B-23971	*Kurthia* sp.	Dry	—	—
Southwest Asia	NRRL B-41004	*B.* sp. 3	Mucoid	Than	Smooth
Southwest Asia	NRRL B-23970	*B.* sp. 3	Dry	Thin	Thin
Southwest Asia	NRRL B-23937	*B.* sp. 4	Dry	—	—
Southwest Asia	NRRL B-23938	*B.* sp. 4	Dry	—	—
Southwest Asia	NRRL B-41005	*B. subtilis*	Mucoid	Incomplete	Thin
USA	NRRL B-23980	*B.* sp, 1	Dry	Smooth	Smooth
USA	NRRL B-23975	*B. pumilus*	Dry	Textured	Textured
USA	NRRL B-23983	*B. pumilus*	Dry	Incomplete	Incomplete
USA	NRRL B-23984	*B. pumilus*	Dry	Incomplete	Incomplete
USA	NRRL B-23958	*B.* sp. 1	Mucoid	Smooth	Smooth
USA	NRRL B-23959	*B.* sp. 1	Mucoid	Smooth	Textured
South Korea	NRRL B-23976	*B.* sp. 1	Mucoid	Textured	Smooth
South Korea	NRRL B-23977	*B.* sp. 1	Dry	Smooth	Textured

[a]Species were identified from analyses of the 16S (all species) and/or *gyrA* (*B. subtilis* species group only) genes.
[b]Indicates the geographic origin where the cigarettes were manufactured and packaged.
[c]Indicates type of colony when grown anaerobically on CDC blood agar.
[d]A dash (—) indicates that a pellicle did not form.

other *Bacillus* species sampled on the basis of where they cluster in the 16S phylogeny (Fig. 1). The third species is strain NRRL B-23971, which has a 16S sequence identical to that of a psychrophilic strain (NRRL B-23969) isolated from a bog in Wisconsin and recently deposited into the ARS Culture Collection. Together, these strains constitute a species of *Kurthia* on the basis of their close relationship to *K. sibirica* in the 16S phylogeny (Fig. 1).

A number of strains were typed as *B. subtilis* on the basis of their 16S sequence identities and where they grouped in the 16S phylogeny (Fig. 1). However, members of the *B. subtilis* species group cannot be distinguished from each other on the basis of morphology or 16S sequence divergence alone [2, 24, 27, 28], but the *gyrA* gene has been shown to successfully and reliably distinguish members of the *B. subtilis* species group [9, 24]. A phylogenetic analysis of the *gyrA*

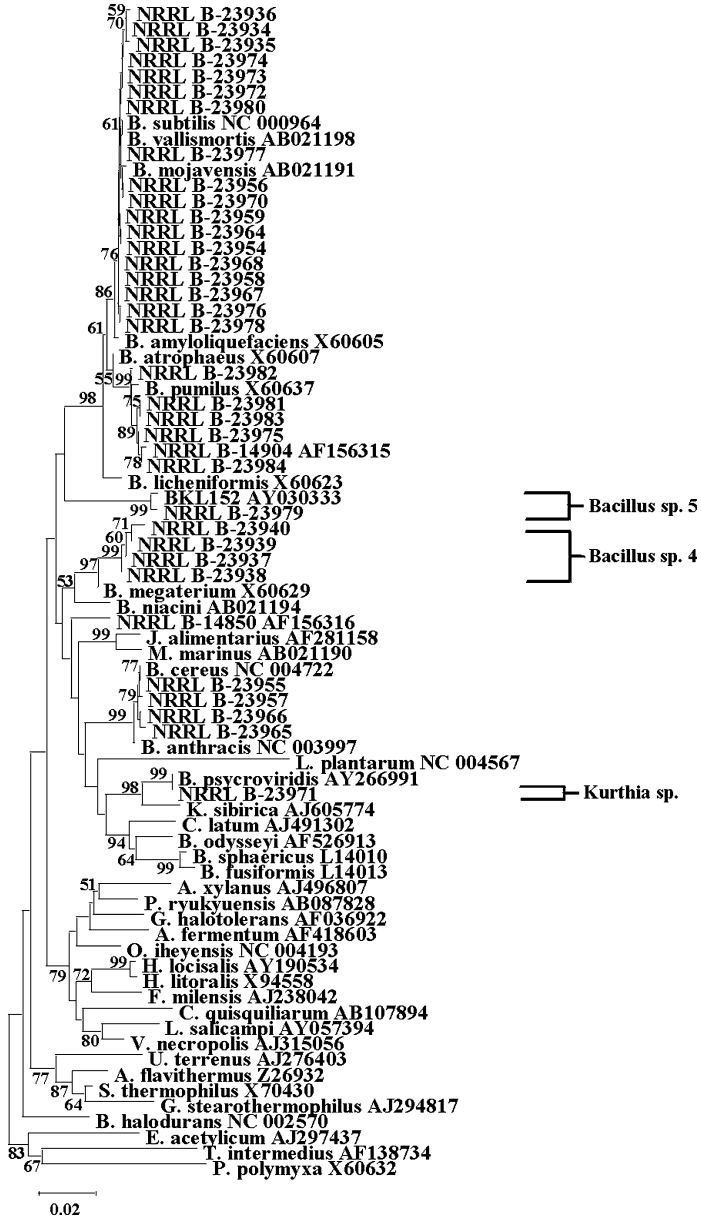

Fig. 1. The 16S rRNA gene phylogeny of bacterial isolates described in this study and previously identified species of Bacillaceae. The numbers along branches represent bootstrap percentages.

sequences for the 22 *B. subtilis* isolates showed that 14 of these strains encompassed 3 new species within the *B. subtilis* group (Fig. 2). Two of the species are closely related to *B. amyloliquefaciens*, while the third is closely related to *B. mojavensis*.

In terms of bacterial species diversity among some of the cigarette brands collected in Iraq, American brands (including the samples collected at Aberdeen Proving Ground, MD) yielded two species (*B. pumilus* and *Bacillus* sp.1), the European brands yielded seven species (*B. cereus, B. pumilus, B. subtilis, Bacillus* sp. 2, *Bacillus* sp. 3, *Bacillus* sp. 4, and *Bacillus* sp. 5), while the Southwest Asian brands yielded six species (e.g., *B. cereus*, *B. subtilis*, *Bacillus* sp. 1, *Bacillus* sp. 3, *Bacillus* sp. 4, and *Kurthia* sp.).

Microbiological characterization. The average number of colony-forming units per gram (CFU/g) of sample determined from 3 MPN dilution series was $1.3 \times 10^6 \pm 1.1 \times 10^6$ for the American brands, $2.6 \times 10^6 \pm 2.0 \times 10^6$ for the European brands, and $9.9 \times 10^5 \pm 6.4 \times 10^5$ for the Southwest Asian brands. The difference in

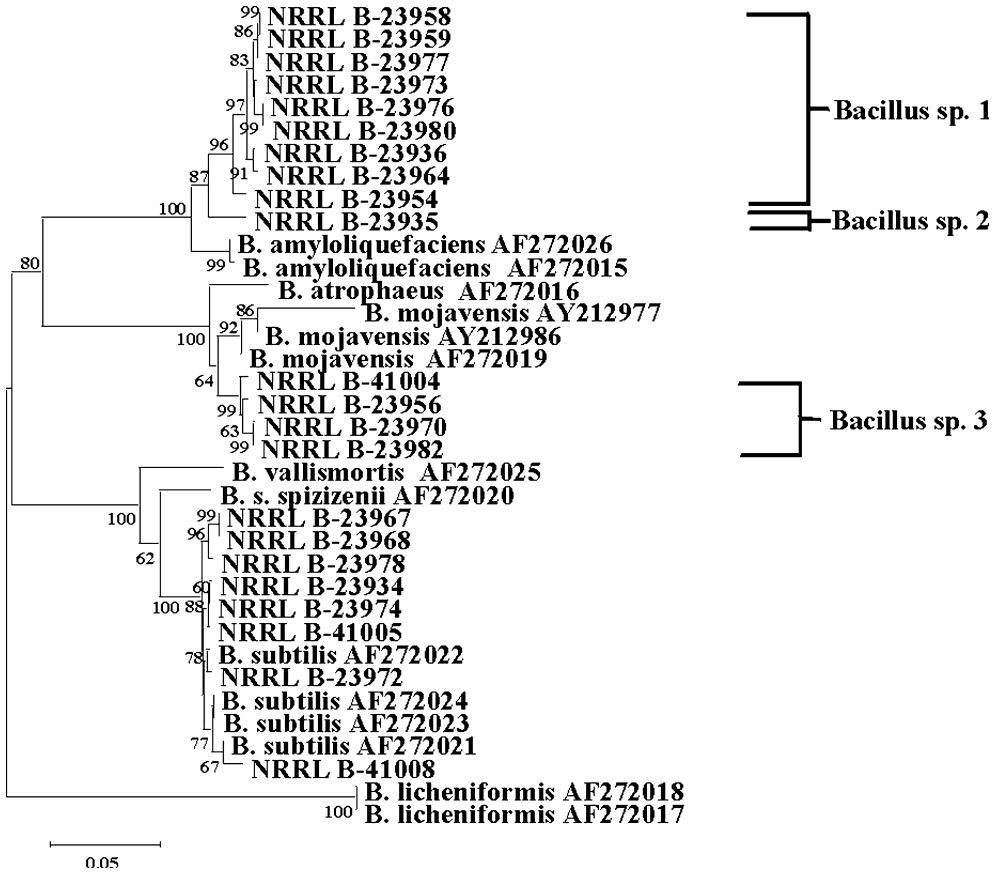

Fig. 2. The *gyrA* gene phylogeny of *Bacillus* isolates described in this study and previously identified members of the *Bacillus subtilis* species group. The numbers along branches represent bootstrap percentages.

CFU/g between the American and Southwest Asian samples was not statistically significant ($P = 0.38$, one-tailed t-test). However, the European sample has a significantly higher bacterial load than either the American ($P = 0.017$, two-tailed t-test) or Southwest Asian ($P = 0.006$, two-tailed t-test) samples. While we did not quantify CFU/g for each species identified, we did find that members of the *subtilis* group were the most commonly encountered organisms overall.

Regardless of the species to which they were assigned, all strains were found to be facultative anaerobes that exhibited the same "dry," flat colony morphology when grown aerobically at 28°C on TGY plates under the conditions described. However, they displayed different colony morphologies when grown anaerobically on blood agar. In this case, some strains produced "dry." flat colonies whereas others produced convex, heavily mucoid colonies. Some species produced only one type of morphology (e.g., *B. cereus* and *B. pumilus*). However, others (e.g., *B. megaterium* and *B. subtilis*) produced both (see also ref. 7).

All strains of *B. subtilis* and the related newly identified species (species 1, 2, and 3) showed the ability to form surface biofilms (Table 1). Most of these isolates showed thin or weak pellicle formation, but in some cases the pellicle was pronounced and smooth. The smooth morphology was clearly the most commonly observed (Table 1). However, a few strains produced textured pellicles that displayed a wrinkled or rough surface (Table 1). The other species examined either did not form pellicles at all (e.g., the newly identified species 4 and 5) or only possessed 1 or 2 strains that had the ability to form biofilms under the conditions examined (i.e., *B. cereus*, *Bacillus* sp. 4, and *B. pumilus*). Two *B. cereus* strains were observed to produce pellicles in anaerobic culture only, but the pellicles were extremely thin and fragile and only covered a small part of the surface. On the other hand, one strain of *B. pumilus* was found to produce a pronounced and textured pellicle in both aerobic and anaerobic culture. Similarly, the sole strain of *B. megaterium* also produced a strong pellicle, but it had an unusual cobweb-like morphology.

Discussion

Few studies have been published on bacterial species diversity of tobacco products [11, 17, 20, 26, 29]. Those that have been published have found that gram-positive bacteria are the most frequently isolated, especially members of the genus *Bacillus*. In the cigarettes that we examined in our study, we also found several species of *Bacillus*, with members of the *B. subtilis* species group being the predominant organisms recovered. We also found that the total bacterial load of the European sample was substantially higher than either the American or Southwest Asian samples. However, the species diversities in the European and Southwest Asian samples were similar (7 and 6 species, respectively), while the American sample had only 2 species (Table 1). The differences in species composition between samples might be explained by differences in tobacco production between the United States versus Europe and Southwest Asia that could result in distinct microbial species that remain viable in cured tobacco post-packaging. Of course, the large distances between these geographic regions could also partially account for differences in species composition between samples.

As in previous studies involving members of the *B. subtilis* group [24, 27, 28], we did not find any phenotypic characters that distinguish these new species from *B. subtilis*. Yet, we did find that many of the *B. subtilis* group isolates that we obtained produced mucoid colonies when grown anaerobically under the described culture conditions and that most of these isolates were able to form biofilms. The ability to form a biofilm has been previously reported for other *B. subtilis* strains [6, 14], though what has been described is somewhat different than what we observed. Branda et al. [6] suggested that laboratory-derived strains of *B. subtilis* characteristically form thin, weak biofilms and that colony morphology on agar plates mirrors pellicle morphology in liquid culture (i.e., isolates that produce wrinkled colonies on agar also produce wrinkled pellicles in liquid culture). They also suggested that thin, smooth pellicles are characteristic of laboratory strains whereas pronounced, textured pellicles are characteristic of wild-type strains. In contrast, we found that the morphology of the surface biofilm did not mirror agar colony morphology and that the textured pellicle type was relatively uncommon among the isolates we obtained.

We also found that biofilm formation was generally more pronounced under anaerobic conditions than under aerobic ones (Table 1). Hamon and Lazazzera [14] suggested that *B. subtilis* biofilm formation is an adaptation to surviving desiccation or environmental stress and will occur if sufficient nutrients are available to maintain growth. In *P. aeruginosa* [10,15] and *Staphylococcus epidermis* [13, 19], biofilm formation is associated with the mucoid colony phenotype. In contrast, the mucoid colony phenotype was not associated with the biofilm-forming ability of the isolates in our study. However, these isolates did show a stronger propensity to form biofilms under anaerobic conditions. Similarly, *P. aeruginosa* undergo anaerobic metabolism in cystic fibrosis patients, especially in the later stages of the disease when mucous build-up is substantial [10,15].

As stated at the beginning of this report, many of the isolates that we characterized belong to species that have been implicated in pulmonary disease. However, it is unknown at this time if exposure to these species contributes to the AEP contracted by U.S. servicemen. Of course, it is entirely possible that AEP is unlinked to the microbial population present in cigarettes and instead is the result of exposure to a different source. In fact, U.S. servicemen who contracted AEP reported significant exposure to airborne dust and sand [1]. Interestingly, *B. subtilis*, the most common isolate in our study, is known to cause hypersensitivity pneumonitis and extrinsic allergic alveolitis [12, 16, 37] and is commonly found in sand and soil. Thus, it would be worthwhile to determine if isolates belonging to this species or others in the *B. subtilis* group trigger an IgE-mediated immune response as seen in AEP.

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