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Terrestrial microorganisms at an altitude of 20,000 m in Earth's atmosphere

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Abstract

A joint effort between the U.S. Geological Survey's (USGS) Global Desert Dust and NASA's Stratospheric and Cosmic Dust Programs identified culturable microbes from an air sample collected at an altitude of 20,000 m. A total of 4 fungal (*Penicillium* sp.) and 71 bacteria colony-forming units (70 colonies of *Bacillus luciferensis* believed to have originated from a single cell collected at altitude and one colony of *Bacillus sphaericus*) were enumerated, isolated and identified using a morphological key and 16S rDNA sequencing respectively. All of the isolates identified were spore-forming pigmented fungi or bacteria of terrestrial origin and demonstrate that the presence of viable microorganisms in Earth's upper atmosphere may not be uncommon.

1. Introduction

Our group at the USGS has been studying long range atmospheric dispersion of microbes in Earth's atmosphere. This research has demonstrated that many species of bacteria and fungi can survive transoceanic transport through the atmosphere (Griffin et al., 2001, 2003). This ability of dustborne microbes to be transported vast distances horizontally in Earth's atmosphere sparked an interest in investigating vertical dispersion.

Mechanisms of vertical transport of microbes into Earth's upper atmosphere include storm activity over land and sea, volcanic activity, impact events and human activity such at weapons testing and spacecraft launches (Hall and Brunch, 1965; Bucker and Horneck, 1969; Simkin and Siebert, 1994; Kring, 2000; Griffin et al., 2002). Through these various mechanisms, microbes could be moved to any of Earth's atmospheric layers to include near Earth space (Figure 1). While the number of studies in the field of high altitude microbiology is limited in scope in comparison to other microbiology fields, there has been a historical record of interest. Louis Pasteur in his quest to dispel the theory of 'Spontaneous Generation' found microorganisms in atmospheric samples taken atop mountains (Pasteur, 1861). A number of European scientists in the late 1800's and early 1900's used balloons to study atmospheric microbiology and reported the recovery of fungi and spore forming pigmented bacteria from various altitudes within the troposphere (Cristiani, 1893; Harz, 1904; Flemming, 1908; Hahn, 1909). Atmospheric research in the 1930's using a balloon to collect a sample as it descended from 21,000 to 11,000 m, recovered 10 spore forming microorganisms, 5 *Bacillus* sp. and 5 fungi (one *Penicillium* sp., one *Macrosporium* sp., one *Rhizopus* sp. and two *Aspergillus* sp.) (Rogers and Meier, 1936). In the 1970's, meteorological rockets equipped to take high altitude air samples recovered viable bacteria and fungi from an altitude of 48,000 to 77,000 m (Imshenetsky et al., 1978). This research project also noted that 'A greater number of microorganisms have been registered in the mesosphere during dust storms than in the absence of strong winds.' (Imshenetsky et al., 1978). Fun-

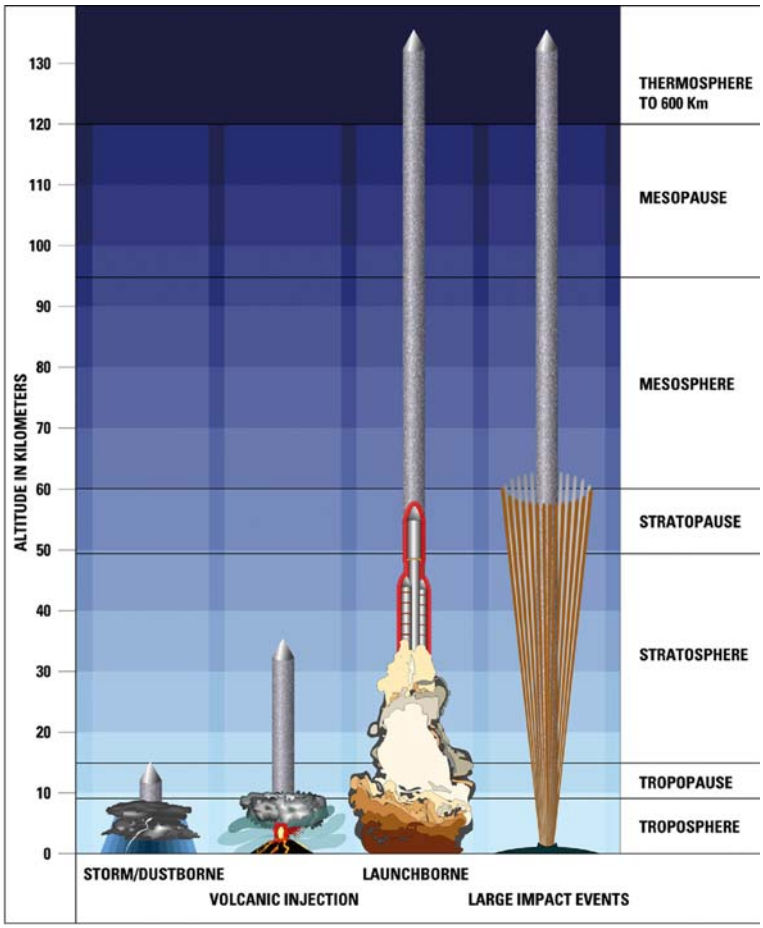

Figure 1. Cartoon depicting means of microbial movement into the upper atmosphere. Storm activity has the capability of lifting microorganisms to the tropopause and possibly beyond. Large volcanic eruptions can lift large numbers of microorganisms into the stratosphere. Spacecraft can lift microorganisms the upper limits of Earth's atmosphere and beyond. Large impact events can eject large quantities of debris and microorganisms to space. Figure artwork by Betsy Boynton, USGS, CCWS, St. Petersburg, Florida.

gal isolates recovered from these extreme altitudes were typically more resistant to ultra violet light (UV) inactivation or freeze/thaw exposures than the same species collected from terrestrial environments (Imshenetsky et al., 1978, 1983). Of the species of fungi collected from extreme altitudes, *Aspergillus* sp. were more resistant to stress (UV and vacuum exposure) than other genera (Lysenko, 1980).

Our ground and ship based lower atmosphere studies have identified a diverse group of both bacteria and fungi capable of surviving transoceanic transport in the atmosphere (Griffin et al., 2001, 2003). The majority of isolates obtained from these studies are spore forming microorganisms (e.g. fungi and bacteria of the genera *Bacillus*), bacteria of high guanine (G) and cytosine (C) content (high G/C content DNA is more resistant to UV damage relative to low G/C content), and/or are pigmented (cell wall pigments also impart some degree of UV resistance) (Singer and Ames, 1970; Sundin and Jacobs, 1999; Setlow, 2001; Nicholson et al., 2002). Relative to this data a number of questions arose; (1) could we isolate microorganisms from high altitudes in the atmosphere? (2) if we obtained isolates, how would they compare to what has been observed historically? and (3) using the same culture media and techniques employed in our low altitude studies how would these isolate groups compare? With the assistance Mike Zolensky and Jack Warren of NASA (Johnson Space Center, Houston, Texas)

an air sample was obtained from a sustained flight at an altitude of 20,000 m. This report summarizes the identification of viable microorganisms in that sample.

2. Material and methods

2.1 *Sample device and preparation*

An impacter device was sent to our laboratory by the NASA Cosmic Dust Group. This device is a small impacter plate, which is mounted on a post within a sealed housing. This sealed housing is composed of a gasket-lined lid, which is secured to a base plate by four screws. Upon receipt of the device the impacter plate (plastic) was duplicated using steel so that the entire unit could withstand autoclaving (Figure 2). The unit was autoclaved along with an aliquot of glycerol for 15 minutes at 121 °C. After autoclaving, the face of the impacter plate was coated with a fine layer of glycerol. The impacter unit was then assembled and placed in its sealed housing, wrapped in aluminum foil and autoclaved again for 15 minutes at 121 °C. To account for contamination of the autoclaved glycerol a small aliquot was spread onto R2A media (Fisher Scientific, Atlanta, GA) and incubated for one week. No growth was noted on this negative control. All work was performed within a laminar flow hood using sterile technique.

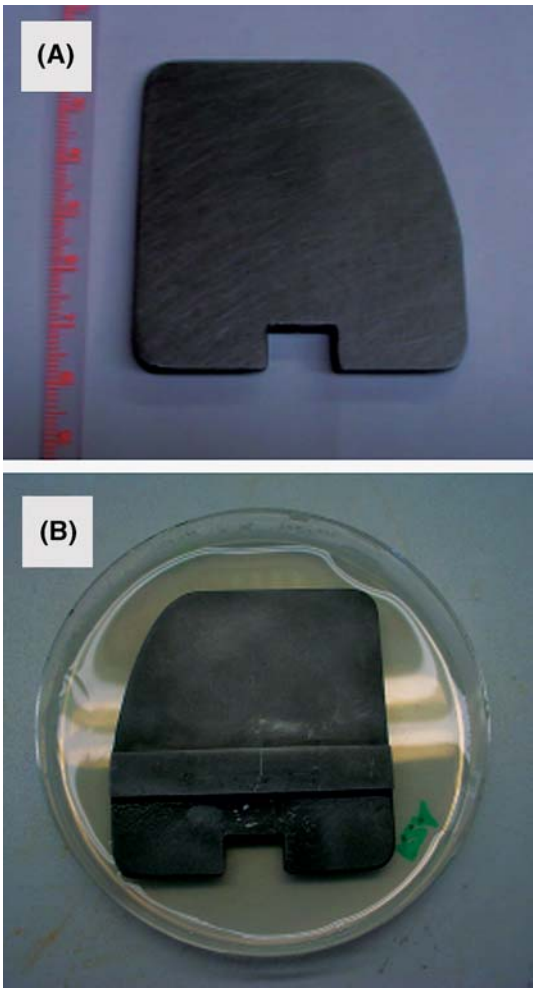

Figure 2. Impacter plate next to centimeter scaled ruler (Panel A) and inoculated onto nutrient agar (R2A media, Panel B).

2.2 *Sampling*

After the impacter device was assembled and sterilized it was shipped to NASA's Cosmic Dust Group for flight routing. The device was mounted within a collector housing located on the underside of a Lockheed Martin ER-2 (Figure 3). The collector was then flown (sortie # 039023) and exposed to the atmosphere for 2.5 hours (opened 1819 Zulu and closed 2049 Zulu) while the plane flew over land between Albuquerque, NM and Edwards Air Force Base, CA at a sustained altitude of 20,000 m.

2.3 *Sample analysis*

After the U.S. Geological Survey laboratory received the sample apparatus, the impacter plate was removed from the housing and base post in a laminar flow hood. A sterile glass spread stick was run across the face of the plate and then inoculated onto a petri dish containing R2A media using spread plate technique. The impacter plate was then placed face down on another petri dish containing R2A media. Both and plates were then incubated at room temperature (~23 °C) for 24 hours (Figure 2). After 24 hours of incubation the impacter plate was removed from the plate of R2A. The plate of R2A was then incubated for an additional 24 hours at room temperature (both plates were incubated for a total of 48 hours). After incubation, colonies were counted and then isolated using secondary streaking and later archived at −70 °C in tryptic soy broth.

Figure 3. Lockheed Martin ER-2 high altitude research aircraft.

2.4 *Genetic identification of prokaryotes*

The polymerase chain reaction (PCR) was used for 16S rDNA amplification using a universal prokaryote primer set (Upstream = 5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3′, Downstream = 5′-CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3′) (Grasby et al., 2003). For DNA extraction, bacterial colonies were touched with a sterile pipette tip, and the tip was then used to inoculate 180 µl of lysis buffer recommended for extraction of DNA from gram positive bacteria in a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA. Catalog # 69504). The DNeasy Tissue Kit protocol was followed and purified DNA was eluted in 100 µl of the kit elution buffer. Ten microliters of purified DNA were used for PCR. The PCR master mix recipe per reaction was: 25 µl of HotStartTaq Master Mix (Qiagen Inc., Valencia, CA. Catalog # 203443), 1 µl each of 10 nM upstream and downstream primer (synthesized by Operon Technologies, Inc., Alameda, CA) and 13 µl of distilled H_2O (HotStartTaq Master Mix Kit component). The PCR amplification profile was: 1 cycle for 15 minutes at 95 °C, 40 cycles of [30 seconds at 94 °C, 30 seconds at 45 °C, 3 minutes at 72 °C], one cycle of 10 minutes at 72 °C and hold at 4 °C. PCR amplicons were cleaned and directly sequenced (1 strand, 1 reaction using the downstream primer) by Northwoods DNA, Inc. (Becida, MN). GenBank Blast search (http://www.ncbi.nlm.nih.gov/BLAST/) was used for amplicon/isolate identification. DNA alignments (Clustal W) were performed using Lasergene MegAlign (DNASTAR, Inc. Madison, WI).

2.5 *Fungi identification*

Fungi were identified to the genus level based on morphological characteristics such as spore, hyphae and fruiting body shape. In short, colonies were grown in the presence of light at room temperature until sporulation occurred (3–14 days). Fungal tissue was harvested by placing a piece of clear tape in contact with the colony (sticky side to fungi tissue). The tape/tissue was then transferred to a glass slide (fungal tissue is sandwiched between the tape and the glass over the dye drop) containing a small drop of lactophenol cotton blue (~25 µl. DIFCO BBL, Sparks, MD, Catalog # 261188). The fungus was then identified using light microscopy and a morphological key (St-Germain and Summerbell, 1996).

3. Results

A total of 4 fungi and 70 bacteria colony-forming units (CFU) were isolated from the petri dish which had the impacter plate placed directly on it. The 4 fungi and 6 of the bacteria CFU grew at different locations on the R2A media. The remaining bacteria CFU were grouped together in tight association. One bacterium CFU was recovered from the petri dish, which had been utilized for spread plate technique. All of the bacteria CFU formed small white glossy colonies. The fungi CFU were initially white and turned dark when they started to produce spores. The 4 colonies of fungi were identified as the genus *Penicillium*. Because of their likeness and proximity a subset of the total bacteria CFU was selected for identification. Of the 71 bacterial isolates, the first 6, every 10th and the 71st isolates were selected for identification. Sequences lengths used for GenBank Blast and alignments were a minimum of 588 base pairs. With the exception of the 71st isolate, all identified at 99% sequence homology (complete sequence lengths) via GenBank Blast to a *Bacillus luciferensis* (GenBank accession # AJ419629, a volcanic soil isolate from Candlemas Island, South Sandwich archipelago). The 71st isolate was 99%

homologous to a *Bacillus sphaericus* (GenBank accession # AJ311894). Sequence alignment of the isolates demonstrated a sequence identity of greater than 99.7% between the 13 *Bacillus luciferensis* isolates. The *Bacillus sphaericus* isolate which also grew within the cluster of *Bacillus luciferensis* colonies had a sequence identity range of 94.5–94.7% to those isolates. GenBank accession numbers for these isolates are AY291461 through AY291474. Sequence analysis performed at a later date on an additional 43 of the unidentified bacteria isolates identified all of those isolates as *Bacillus luciferensis*. A sequence alignment of all the *Bacillus* isolates again revealed that all of the *Bacillus luciferensis* isolates had sequence identity greater than 99.7% (54 of 56 sequences were 100% identical). These additional sequences were not placed in Genbank to limit repetition.

4. Discussion

This data demonstrates that viable microorganisms can be collected at high altitudes in Earth's atmosphere using a hard surface impacter. The isolates in this study mirror those obtained from other 'high altitude' studies in that they were pigmented spore forming bacteria or fungi (Imshenetsky et al., 1978; Rogers and Meier, 1936). The degree of sequence similarity between the *Bacillus luciferensis* isolates indicates that these individual colonies probably originated from a single cell collected at altitude. It is interesting that of all the possible *Bacillus sp*. matches, they identified to a volcanic soil isolate. Volcanoes can inject significant amounts of soil (and associated bacteria) into the atmosphere (Figures 1 and 4) and microbial populations of a billion or more cells per gram of soil is not uncommon (Ovreas and Torsvik, 1998; Ashelford et al., 2003).

In summary all the microorganisms isolated were spore-formers. Spores are egg-like vesicles which can protect microorganisms from physical stresses such as ultraviolet light induced DNA damage, desiccation, and extreme temperatures (Setlow, 2001; Saffary et al., 2002). Research has led to the speculation that a spore-forming microbe could survive for extended periods of time in Earth's upper atmosphere as well as interplanetary transport and if shielded within a particle, interstellar transport (Nicholson et al., 2000). As Earth has experienced a number of large-scale impact events through time, it may have served as a seed source to extreme atmospheric altitudes, space, and other planets or moons within our solar system. Given our current state of knowledge in the field of environmental microbiology, having not identified all cultivable microorganisms and only able to culture an estimated 1% of existing populations, our quest to prove the existence of life of extraterrestrial origin may be confounded by the presence of ancient terran microbes or their progeny (Suzuki et al., 1997; Eilers et al., 2000).

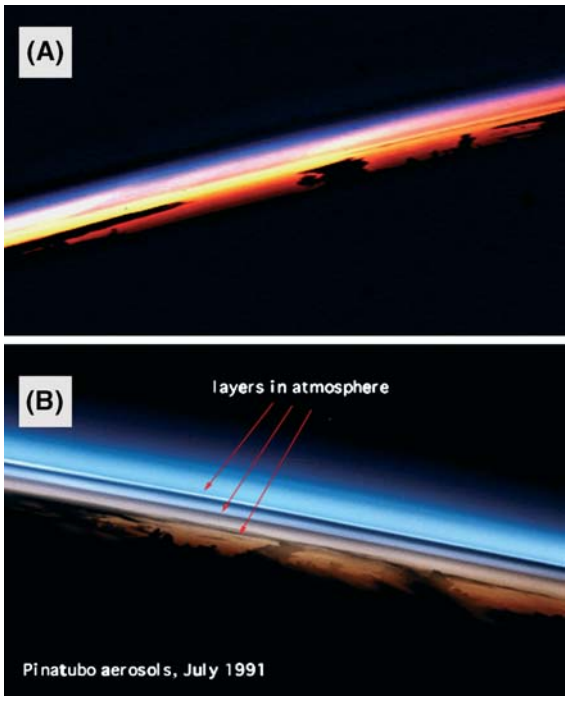

Figure 4. Earth's atmosphere before and after a volcanic event. Images courtesy of Earth Sciences and Image Analysis Laboratory, NASA Johnson Space Center. http://eol.jsc.nasa.gov. Panel A. Clear atmospheric condition showing storm activity in the troposphere/tropopause taken August 30, 1984. Mission STS41D, Roll 32, frame 14. Panel B. Volcanic aerosol layers in stratosphere (altitude ~20–25 km) two months after the Pinatubo eruption taken August 8, 1991. Mission STS043, Roll 22, frame 23.

Current and historic research as outlined in this report has demonstrated the presence of microbes at various altitudes in Earth's atmosphere and the ability of certain microorganisms to survive the physical stresses of these environments. Future collections should expand our understanding of the diversity, distribution and movement of microbes in Earth's upper atmosphere.

Acknowledgements

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