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ORIGINAL PAPER

# Microbial survival in the stratosphere and implications for global dispersal

**David J. Smith · Dale W. Griffin · Richard D. McPeters · Peter D. Ward · Andrew C. Schuerger**

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**Abstract** Spores of *Bacillus subtilis* were exposed to a series of stratosphere simulations. In total, five distinct treatments measured the effect of reduced pressure, low temperature, high desiccation, and intense ultraviolet (UV) irradiation on stratosphere-isolated and ground-isolated *B. subtilis* strains. Environmental conditions were based on springtime data from a mid-latitude region of the lower stratosphere (20 km). Experimentally, each treatment consisted of the following independent or combined conditions: −70°C, 56 mb, 10–12% relative humidity and 0.00421, 5.11, and 54.64 W/m$^2$ of UVC (200–280 nm), UVB (280–315 nm), UVA (315–400 nm), respectively. Bacteria were deposited on metal coupon surfaces in monolayers of $\sim 1 \times 10^6$ spores and prepared with palagonite (particle size $< 20$ μm). After 6 h of exposure to the stratosphere environment, 99.9% of *B. subtilis* spores were killed due to UV irradiation. In contrast, temperature, desiccation, and pressure simulations without UV had no effect on spore viability up through 96 h. There were no differences in survival between the stratosphere-isolated versus ground-isolated *B. subtilis* strains. Inactivation of most bacteria in our simulation indicates that the stratosphere can be a critical barrier to long-distance microbial dispersal and that survival in the upper atmosphere may be constrained by UV irradiation.

**Keywords** Stratosphere · Natural selection · Dispersal · Spores · Aerobiology

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## 1 Introduction

Reports of viable bacteria and fungi in the stratosphere have become increasingly common (Imshenetsky et al. 1978; Lysenko 1980; Harris et al. 2002; Wainwright et al. 2002; Narlikar et al. 2003; Griffin 2004, 2008; Shivaji et al. 2006, 2009; Yang et al. 2008a, b; Smith et al. 2009). Microbes are extremely abundant in soil, and some have adaptations for aerial dispersal (Papke and Ward 2004; Kellogg and Griffin 2006). While limited mixing at the tropopause boundary

restricts most airborne microbes to lower altitudes, a wide range of mechanisms can transport aerosols (or biological cells) from the troposphere to the stratosphere, including the following: volcanic eruptions (Jacob 1999); Brewer–Dobson atmospheric circulation (Wallace and Hobbs 2006); dust storms (Kellogg and Griffin 2006); monsoons (Randel et al. 2010); electrostatic forces created by thunderstorms (Dehel et al. 2008) and rocket launches (Griffin 2004). It is unknown how long microbes can remain aloft in the stratosphere, but aerosol studies have shown it takes months and sometimes years for particulates to eventually return to the surface (Jacob 1999; Wallace and Hobbs 2006). Most stratospheric aerobiology studies have focused only on the characterization of microbes (i.e., identifying species and place of origin), leaving other key ecological questions unaddressed. For example, how long can cells remain viable in the stratosphere? What atmospheric and biological factors ultimately control the survival of cells? Answers to these questions may provide a critical framework for understanding patterns of microbial biogeography and the evolutionary consequences of long-distance dispersal via upper atmosphere pathways.

To address survival, one must consider the absence of nutrients, reduced atmospheric pressure, extreme desiccation, low temperatures, toxic chemical species, and intense solar radiation in the stratosphere. Atmospheric pressure is inversely related to altitude; meaning at the tropopause ($\sim$18 km) atmospheric pressure can be close to 100 mb, whereas in the stratopause ($\sim$50 km) the value is almost two orders of magnitude smaller (Dessler 2000; Wallace and Hobbs 2006). Although relative humidity (RH) levels are difficult to determine, dryness helps define the stratospheric boundary layer with the troposphere. One study across North America and Europe reported average RH levels in the stratosphere at $\sim$23% (Gierens et al. 1999). Meanwhile, daytime temperatures can be as low as $-70$°C in the tropopause and as high as 0°C in the region of maximum stratospheric ozone ($O_3$) where absorption of short-wavelength ultraviolet (UV) light generates heat (Brasseur and Solomon 1986; Jacob 1999; Dessler 2000; Wallace and Hobbs 2006). Understanding UV irradiation is therefore critical to characterizing the stratospheric environment with regard to microbial life. However, generalizing about UV is challenging because conditions vary according to the ozone column (i.e., altitude); latitude; weather; solar zenith angle; and scattering from molecules, aerosols or clouds (Brasseur and Solomon 1986; Daumont et al. 1992; Malicet et al. 1995; Blumthaler et al. 1997; Dessler 2000; Schmucki and Philipona 2002; Kondratyev et al. 2006; Wallace and Hobbs 2006).

Incredibly, microbes that can form endospores (hereafter referred to as 'spores') appear to survive the harsh environmental conditions described above (Imshenetsky et al. 1978; Harris et al. 2002; Wainwright et al. 2002; Narlikar et al. 2003; Griffin 2004; Yang et al. 2008a, b; Shivaji et al. 2006, 2009; Smith et al. 2009). Sporulation offers protection against factors that would otherwise threaten vegetative cells (Nicholson et al. 2002). Spores are a dormant stage in the life cycle of a microbe, but viable cells can germinate and resume activity upon contact with liquid water and nutrients at favorable temperatures (Nicholson et al. 2000, 2002). The bacterium *Bacillus subtilis* was chosen for this study because the responses of its spore to extreme environments have been well documented (Horneck et al. 1994; Nicholson and Fajardo-Cavazos 1997; Nicholson et al. 2000, 2002; Riesenman and Nicholson 2000; Slieman and Nicholson 2000, 2001; Dose et al. 2001; Setlow 2001, 2007). In addition, it is a common soil microorganism that has been found previously in the stratosphere (Smith et al. 2009). Nicholson et al. (2000) have reviewed the main physical and molecular defenses that *B. subtilis* uses to protect and repair biomolecules (e.g., deoxyribonucleic acid (DNA)) during dormancy or germination. In brief, spore cores are dehydrated and surrounded by a highly impermeable coat, reducing desiccation and irradiation stress (Setlow 1995; Riesenman and Nicholson 2000; Nicholson et al. 2002; Ghosal et al. 2005). Genetic resistance to extreme factors has been associated with several features, including the ability to stabilize DNA strands with small acid-soluble proteins (SASP) (Nicholson et al. 2000), active repairs to damaged macromolecules (Setlow 1995; Xue and Nicholson 1996), and the production of photoprotective pigments (Imshenetsky et al. 1979; Nicholson et al. 2002).

A common conclusion in upper atmosphere aerobiology (Imshenetsky et al. 1977; Yang et al. 2008a, b; Shivaji et al. 2006, 2009) is that airborne microbes have higher resistance to environmental extremes than

similar ground strains because the stratosphere has selected for hardier cells (i.e., harsh conditions have preferentially killed off weaker cells). Although intuitive, the claim has not been adequately supported by experimental studies. The first problem with previous microbial resistance studies relates to the simulated UV environment. Imshenetsky et al. (1977) and Yang et al. (2008a, b) used monochromatic 254-nm UV lamps positioned at an arbitrary distance from microbes to evaluate resistance. However, UVC (200–280 nm) sources are a poor approximation of sunlight in natural environments (Xue and Nicholson 1996; Nicholson et al. 2000, 2002) and by focusing only on the 254-nm band those studies failed to address the effect of UVB (280–315 nm) and UVA (315–400 nm) wavelengths, which can also contribute significantly to cell death (Xue and Nicholson 1996; Slieman and Nicholson 2001). Second, many previous resistance studies have been conducted at ground-normal conditions instead of incorporating stratospheric pressure, desiccation, and temperature. Alone, these factors might have little effect on spore viability (Horneck 1993; Dose and Klein 1996; Miyamoto-Shinohara et al. 2006; Osman et al. 2008), but when coupled with UV irradiation the synergistic stress has been shown to amplify the degradation of biomolecules (Nicholson et al. 2000; Saffary et al. 2002; Diaz and Schulze-Makuch 2006). Only Imshenetsky et al. (1977) evaluated survival in stratospheric temperature and pressure regimes, but did so independently of UV irradiation. Third, Yang et al. (2008a, b) and Shivaji et al. (2006, 2009) relied mainly upon genus-level comparisons of microbial resistance, introducing the possibility of significant genetic variation between samples. Finally, the physical distribution of bacterial spores during previous studies might have generated misleading results. To facilitate dispersal, spores tend to clump onto substrates—and oftentimes other spores. Yang et al. (2008a) acknowledged limited success in dispersing spores evenly during UV experiments. Neither Imshenetsky et al. (1977) nor Shivaji et al. (2006, 2009) controlled for spore layering, probably exposing dense microbial suspensions to UV. The protective effect of dead and aggregated cells could have provided a significant barrier to irradiation (Horneck et al. 1994; Xue and Nicholson 1996; Nicholson and Law 1999; Schuerger et al. 2003; Wainwright et al. 2006).

Our approach was to revisit the question of stratospheric microbial resistance with a more robust environmental simulation. We measured the viability of *B. subtilis* in experiments that simulated exposure to a subtropical altitude of 20 km; i.e., below most of the ozone layer. Simulations consisted of one or multiple stratosphere conditions (UV, temperature, pressure, and desiccation) in order to determine how each factor would affect cell survival. Our objectives were fourfold: (1) measure an overall viability fraction to understand the likelihood of long-term microbial survival in the stratosphere; (2) identify critical upper atmosphere environmental factors that limited survival; (3) compare the difference in viability between stratosphere and ground isolates of *B. subtilis*; and (4) evaluate how the presence/absence of dust analog particles affected survival. Collectively, our results addressed the hypothesis that the stratosphere is a barrier to the survival of airborne microbiota during global dispersal events. We predicted that: (1) most spores would be killed during simulations; (2) low pressure and desiccation might contribute to the inactivation of cells, but UV irradiation would be the dominant biocidal factor; (3) greater resistance would be measured in stratosphere-isolated *B. subtilis* since it had demonstrated survivability in situ; and (4) dust particles would shield microbes from harmful UV irradiation during simulations.

## 2 Materials and methods

### 2.1 Microbiological procedures

Isolate "NASA8" from Smith et al. (2009) was a *Bacillus subtilis* strain (GenBank Accession #FJ649342) collected in the stratosphere at 20 km over the Pacific Ocean. The second bacterium used in this study was the ground isolate "WN696", a *B. subtilis* strain (GenBank Accession #AY260858) obtained from W. Nicholson (University of Florida, USA); originally collected from desert basal outcrop on Sentinel Hill, Arizona, by Benardini et al. (2003). On Bacto™ Tryptic Soy Agar (Becton–Dickinson and Co., Sparks, MD, USA), both NASA8 and WN696 displayed growth after 24 h as small, round, white colonies. In liquid Bacto™ Tryptic Soy Broth (Becton–Dickinson and Co., Sparks, MD, USA), growth (12–72 h) was distinguishable by culture turbidity; WN696 was denser than NASA8 over the same incubation period and conditions.

Since the experimental equipment was not designed for free-floating spores, aluminum coupons (2 × 1 × 0.1 cm) previously coated with a chromate conversion film (Iridite 14-2, MacDermid, Inc., Waterbury, CT, USA) were used as microbial substrates (see Schuerger et al. 2003, 2008). Prior to experimentation, spores of NASA8 and WN696 were generated according to the protocol by Mancinelli and Klovstad (2000) and Schuerger et al. (2003). From this stock, spores were diluted in sterile deionized water (SDIW) and quantified using a Spectronic-20 Genesys™ spectrometer (model 4001, Sigma–Aldrich, St. Louis, MO, USA) at 600 nm to achieve optical densities of ~0.32. To create uniform monolayers of spores on the aluminum coupons, quantified suspensions were spotted onto the surfaces in 100-µl aliquots per coupon and left covered for 12 h at standard room temperature (25°C) and pressure (1,013 mb) (STP). The number of bacteria applied onto coupons in 100-µl aliquots was measured at ~$1 \times 10^6$ cells. Before experimental use, doped coupons were uncovered and allowed to dry for 2–4 h in a dark laminar flow hood (NuAIRE Biological Safety Cabinet, Class II Type A/B3, Model NU-602-400, Plymouth, MN, USA). All coupons had been dry-heat sterilized overnight at 130°C and cooled to 24°C before bacteria spores were deposited as monolayers. The coupon preparation procedure was standardized and used for all experiments.

To determine the effect of atmospheric dust particles on the survivability of *B. subtilis* spores, half of the NASA8 and WN696 coupons were mixed with palagonite collected by A. Schuerger (University of Florida, USA) from the Cerros del Rio volcanic field, New Mexico, USA. Elemental composition of the dust analog was measured by energy-dispersive X-ray spectrometry (EDS) (NORAN Systems SIX, Thermo Electron Corp., Waltham, MA, USA). To prepare the fine-grained particles representative of stratospheric dust (Carder et al. 1986; Betzer et al. 1988), granular palagonite was ball-milled for 24 h. Five hundred grams of the crushed palagonite was then passed through a series of dry sieves (200, 75, 45, 32, and 20 µm) using a shaker apparatus (Retsch AS 200, Newton, PA, USA). The sieved dust product was examined with a scanning electron microscope (SEM) (JSM-7500F, JEOL Ltd., Tokyo, Japan) to assess particle sizes and sterilized at 130°C for 24 h prior to use. To create the aliquot for certain NASA8 and WN696 dust-prepared coupons, 0.10 g of dust was added to 10 l SDIW where it dispersed with agitation. Then, 1 ml of that dust solution was combined with 9 ml SDIW and 10 µl spore stock.

## 2.2 UV parameters

The UV model was developed to correspond to the sampling site of NASA8 by Smith et al. (2009); i.e., 20 km altitude at a mid-latitude location (36°N) in boreal springtime. Calculated values were only for direct UV flux at a fixed solar zenith angle of 30°. Bulk solar fluence values were based on measurements from Nimbus-7 Solar Backscatter UV (SBUV) instrument that took solar spectra during an ascent through the stratosphere in January 1979 (McPeters et al. 1984, 1993). In order to account for UV attenuation at the simulated altitude, ozone cross-sections by McPeters et al. (2007) were averaged and converted to absorption coefficient factors, then applied toward a modified Beers law equation:

$$l_{(p)} = F_0^{-s([\alpha * X]+[\beta * P])}$$

where $l_{(p)}$ represented the UV flux, $F_0$ was the extraterrestrial solar flux, $s$ was the relative optical path (solar zenith angle), $\alpha$ was the ozone absorption coefficient (Daumont et al. 1992; Malicet et al. 1995), $X$ was the cumulative ozone above pressure ($P$), and $\beta$ was the Rayleigh scattering coefficient (Bates 1984). Fluence rates were calculated and integrated to provide a total, instantaneous flux rate (in W/m$^2$) for UVA, UVB, and UVC. These modeled values of 82.35, 4.16, and 0.0055 W/m$^2$ for UVA, UVB, and UVC, respectively, became the target fluence rates for environmental simulations (see Table 1). Experimentally, bacteria-doped coupons were exposed continuously to UV, meaning that a 4-day exposure more closely resembled an 8-day exposure in nature, based on cumulative dosage principles.

Two separate UV irradiation systems (described by Schuerger et al. 2003, 2006, 2008) were calibrated to represent the stratospheric illumination at 20 km. One system was located on a laboratory bench, and the second was attached to the hypobaric environmental chamber (see Sect. 2.3). Each system consisted of a xenon-arc lamp (model 6269, Oriel Instruments, Stratford, CA, USA) at a power setting of 1,100 W. Light was passed through a 6-cm water filter to remove intense near-infrared (NIR) photons above

**Table 1** UV fluence rates for experiments

| Spectral ranges (nm) | Modeled UV baseline: *target* values (W/m$^2$) | UV Control (+UV): *actual* values (W/m$^2$) | Stratosphere (+UV): *actual* values (W/m$^2$) |
|---|---|---|---|
| UVA (315–400) | 82.35 | 45.83 | 54.64 |
| UVB (280–315) | 4.16 | 4.16 | 5.11 |
| UVC (200–280) | 0.00550 | 0.00379 | 0.00421 |
| Total UV | 86.52 | 49.99 | 59.75 |

1,100 nm (see Schuerger et al. 2003) and then focused on surfaces where bacterial coupons were exposed. The focus lenses in both systems were located 36 cm above the coupon surfaces. In order to match the target UV fluence rates, the light beams were passed through two opaque materials [a plastic petri dish lid (cat. no. 08-757-11A, Fisher Scientific, Pittsburgh, PA, USA) and a glass petri dish lid (glass-type 7740, Pyrex®Vista™, Corning, Lowell, MA, USA)] chosen to differentially attenuate UVC while allowing a large flux of UVB and UVA to pass (Schuerger unpublished). The conditioned UV irradiation was measured and calibrated with two different spectrometers. High-resolution spectral scans were measured with a model OL754 UV spectrometer from Optronic Laboratories, Inc. (Orlando, FL, USA). A handheld UV measuring system for UVB and UVA fluence rates (model IL1400A, International Light, Newburyport, WA, USA) was cross-calibrated with the OL754 unit and used for routine calibration during individual experiments.

### 2.3 Stratosphere simulation

Simultaneous experiments were conducted to understand the independent contribution of various stratospheric conditions (high UV irradiation, low temperature, extreme desiccation, and low pressure) on the viability of *B. subtilis* spores. Time steps of 6, 12, 24, 48, and 96 h were used. Each experiment had coupons prepared with strain NASA8 or WN696. Coupons were further divided into two separate sets hereafter referred to as "dust" and "dust-free" treatments (see Sect. 2.1). In total, there were five distinct experimental groups (see Table 2), each containing triplicate coupon samples: "Stratosphere (+UV)", "Stratosphere (−UV)", "UV Control (+UV)", "Temperature Control (−UV)", and "Ground Control (−UV)". The Stratosphere (+UV) coupons were inside the hypobaric environmental chamber and exposed directly to UV irradiation; the Stratosphere (−UV) coupons were also inside the hypobaric chamber but completely shielded from UV

**Table 2** Description of experimental groups in stratosphere simulation

| Name | Location | *T* (°C) | *P* (mb) | RH (%) | UV (W/m$^2$) | Purpose |
|---|---|---|---|---|---|---|
| Stratosphere (+UV) | Hypobaric environmental chamber | −70 (± 3) | 56 | 10–12 | UVA: 54.64<br>UVB: 5.11<br>UVC: 0.00421 | Full stratosphere simulation with all environmental variables applied |
| Stratosphere (−UV) | Hypobaric environmental chamber | −70 (± 3) | 56 | 10–12 | Absent | No UV in order to measure effect of stratospheric *T*, *P*, and RH |
| UV Control (+UV) | Laboratory bench | 24 | 1013 | 45 | UVA: 45.83<br>UVB: 4.16<br>UVC: 0.00379 | Standard *T* and *P* experiment to measure effect of stratospheric UV, alone |
| Temperature Control (−UV) | Freezer | −70 | 1013 | 10–20 | Absent | Standard *P* experiment with no UV to measure effect of stratospheric *T* and RH |
| Ground Control (−UV) | Laboratory bench | 24 | 1013 | 45 | Absent | Control experiment with no UV, standard *T*, *P*, and RH |

light; the UV Control (+UV) coupons were irradiated like Stratosphere (+UV) coupons, but remained on the laboratory bench at STP conditions (1,013 mb, 24°C, and 45% RH); the Temperature Control (−UV) coupons were placed in a −70°C freezer in the laboratory (RH levels were 10–20%), and the Ground Control (−UV) coupons were located on the laboratory bench, wrapped with aluminum foil and kept at STP conditions. Each experiment was repeated under identical conditions to provide a total of six replicates for both NASA8 and WN696 treatments.

A hypobaric chamber described by Schuerger et al. (2008) (Fig. 1) was used to create conditions of −70°C (±3°C), 10–12% relative humidity (RH), atmospheric pressure of 56 mb, and a gas composition of $N_2$ (78.08%), $O_2$ (20.95%), Ar (0.93%), and $CO_2$ (0.038%) (Boggs Gases, Titusville, FL). Bacterial coupons were loaded into the hypobaric chamber in plastic petri dishes that sat directly on the upper surface of a liquid nitrogen ($LN_2$) thermal control plate that was used to regulate the temperature of the samples. Instantaneous UV fluence rates for the coupon surface were averaged prior to experimental runs. Stratosphere (+UV) coupons were not exposed to UV light until the hypobaric chamber had equilibrated at the desired set points for temperature and pressure (typically 15–20 min after closure and pump-down). Bacteria-doped coupons were placed randomly within the UV light beam.

Immediately following the environmental simulations, the numbers of viable spores per coupon were enumerated using the Most Probable Number (MPN) method described by Mancinelli and Klovstad (2000) and Schuerger et al. (2003, 2006). In brief, bacterial spores were resuspended in sterile plastic tubes with 10 ml SDIW and vortexed with 1 g of heat-sterilized (24 h at 130°C) silica sand for 2 min. Next, 1 ml of vortexed spore suspension was processed through tenfold serial dilutions with SDIW. Sixteen separate 20-µl volumes from the six dilution steps ($10^{-2}$ through $10^{-7}$) were added to 180 µl of TSB and arranged in a 96-well microtitre plate. The 96-well microtitre plates were incubated at 30°C for 36 h in an InnovaC230 incubator (New Brunswick Scientific, Edison, NJ, USA), and individual wells were visually scored for either positive or negative growth, providing an estimated number of viable cells (see Mancinelli and Klovstad 2000). The minimum detection limit of the assay was 90 spores per coupon.

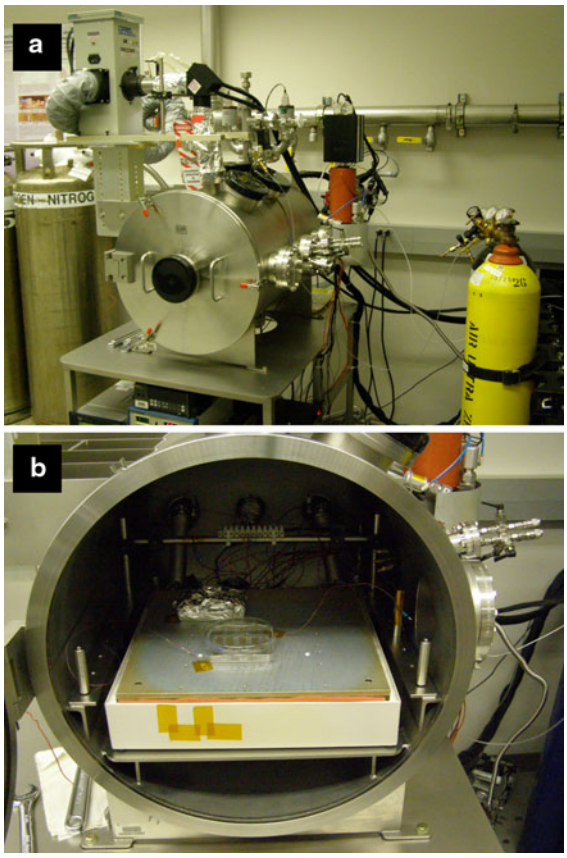


**Fig. 1** Hypobaric environmental chamber used for stratosphere simulation experiments. **a** Temperature, UV light, and gas composition were regulated by liquid nitrogen ($LN_2$), xenon-arc lamp source, and a mass control unit, respectively (pictured from *left* to *right*). **b** Chamber door ajar, revealing Stratosphere (+UV) and Stratosphere (−UV) treatments on $LN_2$ cold plate. Stratosphere (+UV) coupons are located in foreground underneath materials that attenuated UV light close to target fluence rates. Stratosphere (−UV) coupons are in background, completely shielded from UV by aluminum foil. Wiring for thermocouples and RH sensors can be seen

## 2.4 Statistical analysis

The MPN data were log-transformed and analyzed with the statistical program R version 2.3.1 (The R Foundation for Statistical Computing, Vienna, Austria, 2006) at a 95% confidence level. Data were subjected to one-way permutations (Kruskal–Wallis rank-sum test) to compare differences in means across time among individual treatment groups. Wilcoxon rank-sum permutation tests (with a continuity correction) were used to measure differences across species- and dust-specific treatment groups.

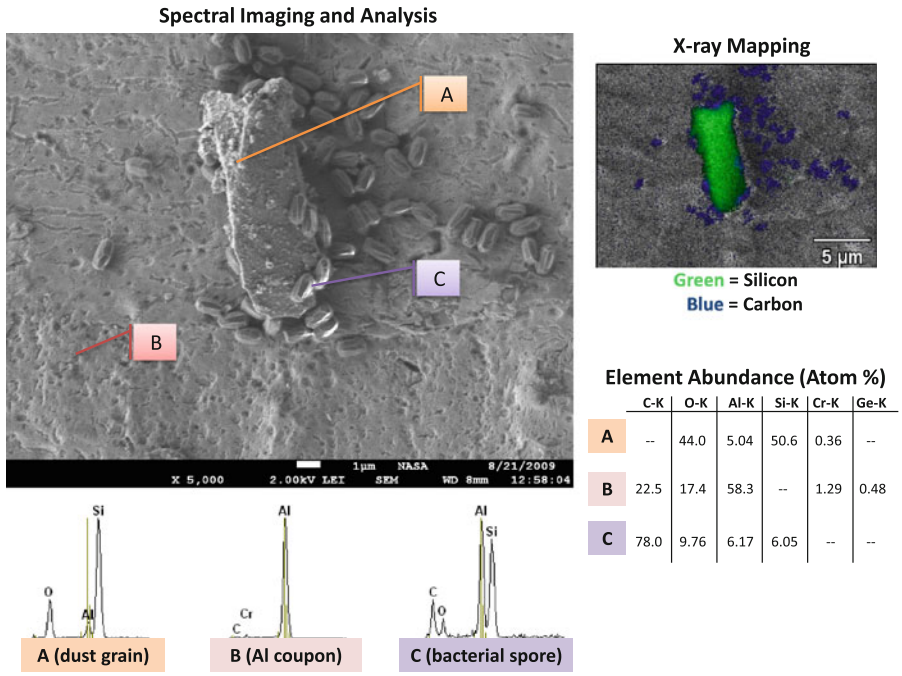


**Fig. 2** Energy-dispersive X-ray spectrometry analysis performed on bacterial coupons. Spectra and elemental abundance of dust (*point A*), coupons surfaces (*point B*), and spores (*point C*) were measured. X-ray mapping highlights dominance of silicon and carbon in palagonite dust analog and bacterial spores, respectively

## 3 Results

Energy-dispersive X-ray spectrometry and scanning electron microscopy (SEM) (Figs. 2, 3) were used to characterize bacterial coupons. Spectral analysis of the palagonite dust yielded an elemental composition of 50.6% silicon, 44.0% oxygen, 5.04% aluminum, and 0.36% chromium (see point A; Fig. 2). Baseline spectra were taken on the Iridite-treated aluminum coupon (point B) and bacterial spores (point C), calculating a relative abundance of aluminum (58.3%) and carbon (78.0%), respectively. X-ray mapping showed the distribution of spores on dust coupons by highlighting the silicon in dust and carbon in spores as two distinct colors. Scanning electron microscopy confirmed that the bacterial spores were dehydrated (Fig. 3a) from the coupon preparation method and generally measured 1 µm lengthwise. Dust particles (Fig. 3b) on palagonite-treated samples were largely diluted on the coupon surface and stood out as prominent features compared with bacterial spores. Particle shape and size varied, but no dust grain was found to be larger than 20 µm in diameter. Spores attached readily to the surface of dust grains but did not appear fully embedded within the microstructures. Still, SEM images suggest some spores could have been shaded from UV light on the underside of the dust grain. Clumping of spores was minimal and seemed to be more prevalent on the dust-treated coupons than on the dust-free coupons. Where clumping did occur, it was observed to be horizontal (i.e., no vertical layering of cells).

Section 2.2 explained how the target UV fluence rates for environmental simulations were calculated from a modified Beers law equation with data input from Nimbus-7 (SBUV) (McPeters et al. 1984, 1993) and the 20-km sampling site by Smith et al. (2009). That *modeled* UV baseline is compared with *actual* UV fluence rates in Fig. 4. Data for the latter were taken with the high-resolution OL754 UV spectrometer at 1-nm intervals across four random locations within the beam area. The UV Control (+UV) experiment values were 0.00379, 4.16, and 45.83 W/m$^2$ in UVC, UVB, and UVA, respectively. Ultraviolet fluence rates for the Stratosphere (+UV) experiment were nearly identical: UVC, UVB, and UVA totaled 0.00421, 5.11, and 54.64 W/m$^2$, respectively. In both (+UV) experimental setups, the total UV was slightly lower than the modeled values because the light attenuation disproportionately lowered the UVA flux in order to achieve closer-to-target UVC and UVB values.

*Bacillus subtilis* strains NASA8 and WN696 exhibited similar responses to the stratospheric

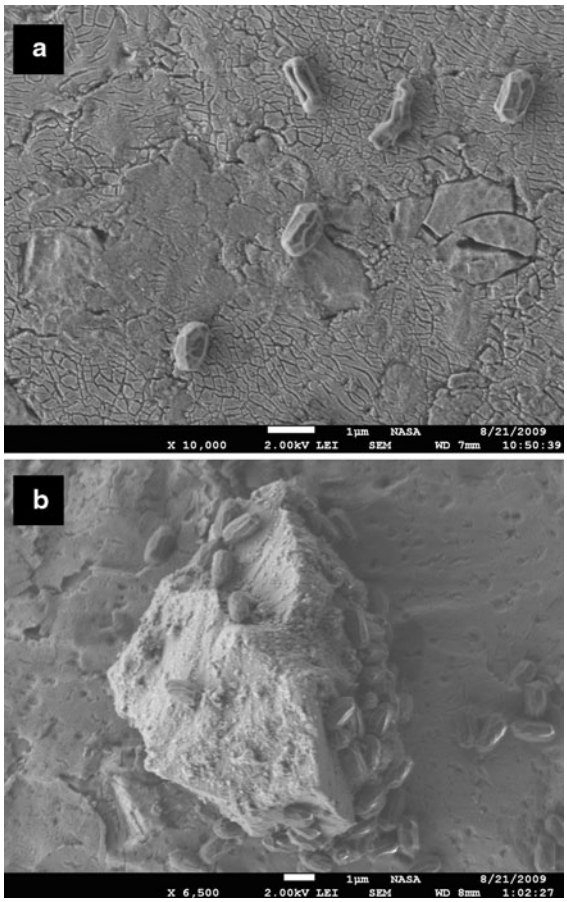


**Fig. 3** Scanning electron microscopy. Monolayers of dehydrated *Bacillus subtilis* spores (*ovoid shaped*, 1 μm) are visible on coupons used for experiments. Number of spores per coupon was estimated at $1 \times 10^6$. **a** Typical distribution of spores on dust-free coupon. **b** Example of dust-treated coupon where spores can be seen attached to a prominent particle

simulations (Fig. 5). The drop in viable spores overtime for UV-exposed (+UV) samples was approximately four orders of magnitude (99.9%), without regard to isolate or dust treatment [Stratosphere (+UV) coupons $P < 0.001$; UV Control (+UV) coupons $P < 0.001$]. While this was an extreme decline from a starting value of $\sim 1 \times 10^6$ cells, the spore die-off was actually incomplete: Both NASA8 and WN696 coupons maintained hundreds to thousands of viable spores through the last time step of 96 h. Even though the difference between the number of viable NASA8 and WN696 spores in the UV Control (+UV) experiment was generally higher than in the Stratosphere (+UV) experiment (except for WN969 dust-treated samples), most of the means had overlapping standard errors. The number of viable spores in Stratosphere (+UV) coupons compared with UV Control (+UV) coupons was calculated as non-statistically significant in each case ($P = 0.0749$–$0.714$).

Rapid loss in viability for both isolates occurred in the first 6 h of experimental exposure and was typically three orders of magnitude. Compared with the non-UV irradiated treatments [Stratosphere (−UV), Temperature Control (−UV), and Ground Control (−UV)], this difference was significant ($P < 0.001$). During subsequent time steps (12–96 h), there was an occasional increase in the number of viable spores, but the data were within (or close to) standard errors of the means. Also between 12 and 96 h, the decrease in spore viability was more moderate compared with the first 6 h of exposure, changing only 1–2 orders of magnitude. Interestingly, dust did not significantly affect spore viability (NASA8 coupons $P = 0.737$–$0.817$; WN696 coupons $P = 0.558$–$0.901$). In every environmental treatment group and with both isolates, spores on dust coupons declined as described earlier. Controls (−UV) for both dust and dust-free samples indicated that pressure, temperature, and desiccation had no effect on the survival of NASA8 or WN696 ($P > 0.05$); all cell counts were within one-half order of magnitude relative to the starting points of the assay.

## 4 Discussion

Motivation for this study was to evaluate the tolerance of microorganisms to stratospheric environmental conditions. We used microbes collected during a trans-Pacific sampling flight at 20 km (Smith et al. 2009) and simulated the stratosphere environment in a hypobaric chamber to address whether the cells were actually capable of a long-term stay in the upper atmosphere. Stratosphere-isolated NASA8 was compared with the ground-isolate WN696 to look for evidence of natural selection and to understand what physical factors were limiting cell viability in the upper atmosphere. Surprisingly, NASA8 and WN 696 had almost identical responses to the simulation: dying rapidly when exposed to UV irradiation and ending with significantly fewer (99.9%) viable cells after 96 h. Running simultaneous experiments with one or more stratospheric factors allowed the effect of individual environmental variables to be measured.

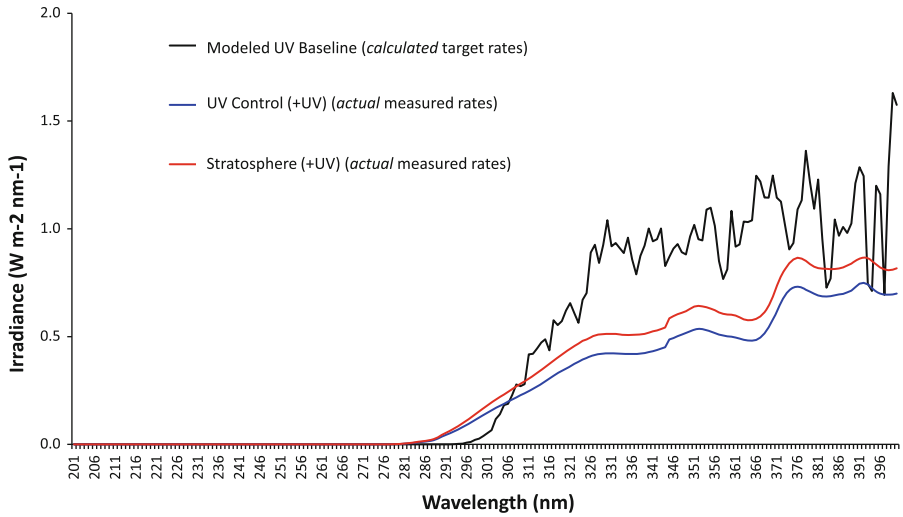


**Fig. 4** Ultraviolet light values. Compilation of modeled (*target*) and measured (*actual*) UV fluence rates from 200 to 400 nm. Baseline values from Nimbus-7 SBUV are depicted in black; UV Control (+UV) values are depicted in *blue*; Stratosphere (+UV) values are depicted in *red*

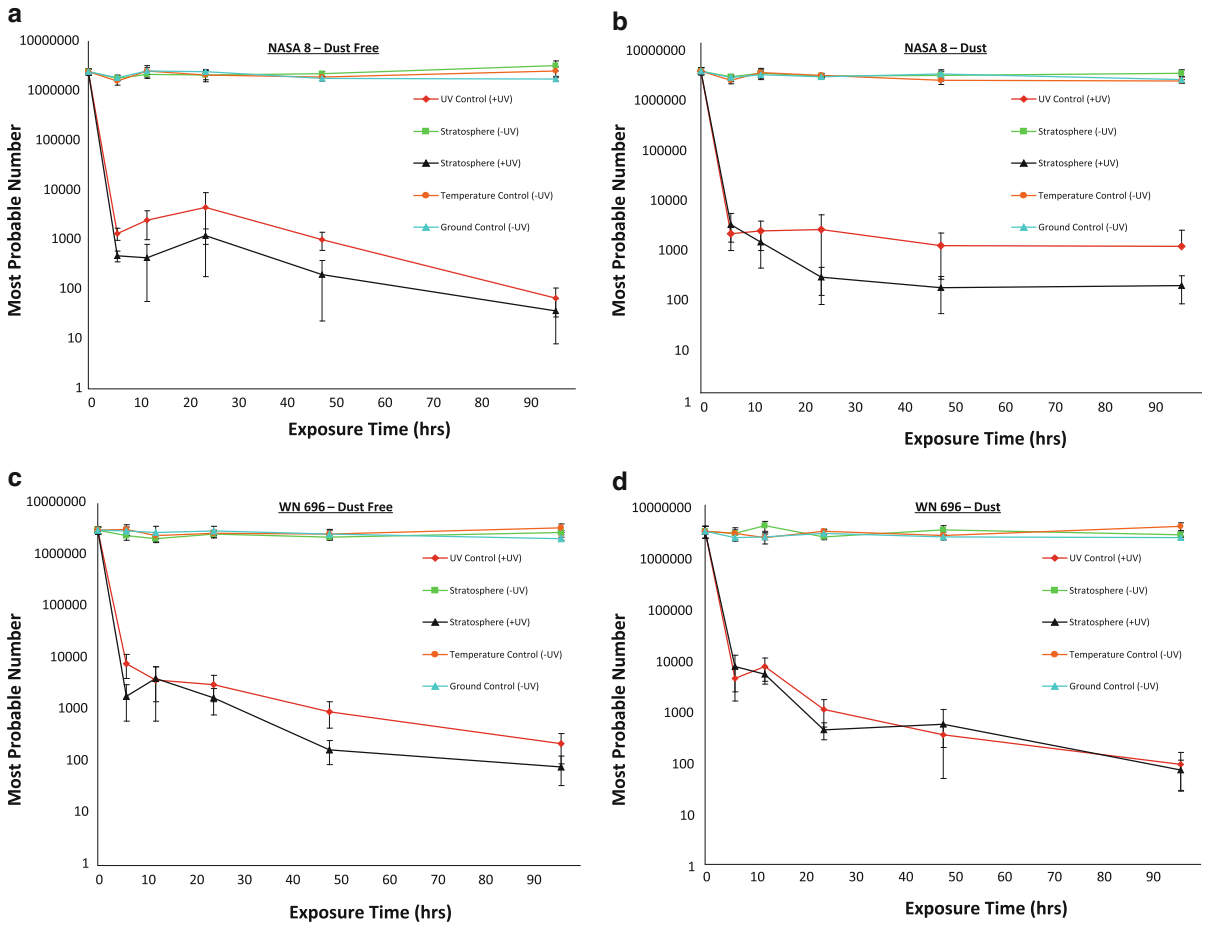


**Fig. 5** Simulated stratosphere experiments. Viability results (MPN) from $t = 0$–96 h. Minimum detection limit of enumeration assay was 90 spores per coupon. Values are means of six replicates; *bars* represent standard errors of the means. **a** NASA8 dust-free coupons. **b** NASA8 dust coupons. **c** WN696 dust-free coupons. **d** WN696 dust coupons

For example, Stratosphere (+UV) and Stratosphere (−UV) coupons differed only in the UV light treatment and shared identical temperature, pressure, and desiccation parameters. But nearly all Stratosphere (+UV) cells were killed, while spores in the Stratosphere (−UV) experimental group did not decline. This finding demonstrates UV irradiation was the primary biocidal component in the simulation, as predicted prior to experimentation. A similar drop in viability for UV Control (+UV) coupons at STP conditions supports the idea that UV irradiation alone, and not simulated stratospheric temperature, pressure, or desiccation influenced the survival of bacteria. The correlation between Stratosphere (+UV) and UV Control (+UV) coupons implies that combining UV with other extreme environmental conditions did not create a synergistic stress effect on microbes. However, these experiments were conducted only with endospores of two *B. subtilis* strains, which are known to resist many common environmental stress factors. It is plausible that interactive effects of stratospheric UV, pressure, temperature, and desiccation might impact non-spore-forming species to a much greater extent.

Our results suggest that NASA8 could have been among the 0.1% of surviving spores recovered from the stratosphere by Smith et al. (2009). Why, then, did it not outlast its ground-derived counterpart (WN696) in stratosphere simulation experiments? Several studies (Nicholson and Law 1999; Nicholson et al. 2002; Benardini et al. 2003; Fajardo-Cavazos and Nicholson 2006; Osman et al. 2008) have compared the UV tolerance between natural populations and reference microbes and may provide insight when comparing NASA8 and WN696. One possibility is that NASA8 spore characteristics may have been substantially different in a laboratory than in the stratosphere. Nicholson and Law (1999) observed that the UV resistance of a natural *B. subtilis* strain was lost when spores were cultivated in the laboratory, even after one round of growth and sporulation. In other words, spore resistance may depend on the conditions in which cells most recently sporulated (e.g., nutrient high, ground-normal conditions in laboratory incubators), with no resistance legacy carried over from the extreme environment of the in situ niche (Nicholson et al. 2000). The fact that the spore preparation method used in this study was identical for both NASA8 and WN696 would then explain the similar survival patterns. In this scenario, it is possible that the same variance in spore construction (e.g. thicker, higher protein ratio, ability to repair biomolecules, etc.) was equally probable for both strains. Another possibility is that spore survival was not based on genetic resistance but instead physical circumstances. For example, microniches on the aluminum coupon surface could have shaded a small fraction of spores from UV (Osman et al. 2008). Dust was expected to have the same type of protective effect on microbes, yet it did not, possibly because the concentration of the dust was too low. Also, spores that were associated with dust grains were generally stuck to rather than embedded within the particle, meaning that only cells on the underside of large dust grains (>1 μm) could have received some irradiation protection. Yet, even that protection may have been incomplete since UV photons could scatter around the base of a dust particle. After using the same coupons, Schuerger et al. (2003) noted that complete protection was only achieved when spores were encased in pits or scratches on the material surface. It is worth noting that our decrease in spore viability from 12 to 96 h (1–2 orders of magnitude) implies that the shading effect from dust and/or coupon microniches cannot account for *all* surviving spores. Following the rapid inactivation of non-shaded spores, one would expect no change in the viability of cells protected by microniches since low temperature, high desiccation, and low pressure did not have a measurable effect on the bacteria.

In this study, UV rates were based on actual measurements from Nimbus-7 (McPeters et al. 1984, 1993). Instead of trying to simulate diurnal cycles and scattering effects, our bacterial coupons were exposed continuously to UV irradiation at a fluence rate similar to a solar zenith angle of 30°. Obviously, this situation would not occur in nature, but the decision to maintain a constant UV flux was because the light equipment did not allow for ramping of diurnal cycles. Also, spore viability depends on cumulative UV dosage, not the rate of UV exposure. Even if simulations included a dark period, the relief would have coincided with a temperature of −70°C, which is likely too cold for DNA self-repair or cellular activity (Junge et al. 2006). The UV simulation was a close match to the stratosphere at 20 km, with the exception of slightly higher UVB and

slightly lower UVA fluence rates (Table 1). Most spore inactivation in our study was due to UVB and UVA irradiation, not to UVC. For stratospheric altitudes considerably higher than 20 km (i.e., above the ozone layer), one would expect a faster kill of cells due to the action of more potent UVC wavelengths. Despite some of the limitations to our UV parameters described earlier, our simulation improved upon previous resistance studies (Imshenetsky et al. 1977; Yang et al. 2008a, b; Shivaji et al. 2006, 2009) because (1) the UV model was specific to the sampling site of the stratosphere isolate; (2) UV was combined with other environmental factors (temperature, pressure, and desiccation) to weigh the relative effects of each variable; and (3) species-level comparisons of microbial resistance were possible.

By measuring the resistance of *B. subtilis*, we addressed the hypothesis that the stratosphere can act as a population bottleneck to airborne microbes transported globally. While the resistance of stratosphere-isolated NASA8 was not unusual compared with the ground strain WN696, the fact that overall numbers were substantially reduced reveals the enormous potential for directed or random selection in the stratosphere. The amount of biological cells in just1 g of soil is staggering—perhaps $10^9$—and it has been estimated that as many as $10^{21}$–$10^{24}$ cells are lifted into the atmosphere annually (Griffin 2010). In many regards, spore-forming microbes are ideally adapted to endure the physical threats associated with airborne transport. Atmospheric mixing events between the troposphere and stratosphere (Griffin 2004; Dehel et al. 2008; Randel et al. 2010) are particularly intriguing because injecting microbes into the upper atmosphere would disperse species globally. Even if 99.9% are inactivated in the stratosphere by UV irradiation, the surviving $10^{18}$–$10^{21}$ cells still represents a huge amount of biomass dispersed. A proxy for predicting microbial dispersal in the stratosphere could be Mt. Pinatubo aerosols, where global percolation throughout both hemispheres occurred months after the mixing event (Wallace and Hobbs 2006). The potential for that kind of global dispersal, combined with the fact that microbial populations are enormous and fast-reproducing, might help explain why distinct biogeography ranges seem absent with many bacteria (Martiny et al. 2006). Another possibility is that microbes surviving stratosphere transport could represent a substantial change from soil-derived gene pools; landing in distant environments with new niches could activate previously suppressed genes (see Wainwright et al. 2006). In both regards, the upper atmosphere could play a major role in the distribution and composition of common microbial species (see Papke and Ward 2004; Kellogg and Griffin 2006; Martiny et al. 2006).

Future studies should consider extending the duration of UV experiments to test whether longer stratospheric survival may be possible (weeks or months). The oxidative effects of ozone (Broadwater et al. 1973; Komanapalli and Lau 1998; Deguillaume et al. 2008)—one biocidal factor not included in this study—should also be measured in an atmospheric context. Embedding microbes inside aerosolized dust would be a useful way of establishing a more dynamic and realistic stratospheric environment. In our study, spreading the cells in monolayers provided interesting information about the ability of individual spores to resist UV, but perhaps it did not simulate the variety of arrangements cells can have while airborne in the stratosphere. For instance, Harris et al. (2002) and Narlikar et al. (2003) noted that microbes collected from 20 to 41 km were clumped together, which implies that the ability to aggregate could be an important aspect of UV resistance at altitude (Wainwright et al. 2006). Additional stratosphere isolates should also be tested (Imshenetsky et al. 1978; Lysenko 1980; Harris et al. 2002; Wainwright et al. 2002; Narlikar et al. 2003; Griffin 2004, 2008; Shivaji et al. 2006, 2009; Yang et al. 2008a, b; Smith et al. 2009), since conclusions from this study most accurately represent the resistance of *B. subtilis* and similar spore-forming bacteria. When using microorganisms for experiments, it would be beneficial to extract spores directly from the environmental samples, in order to ensure that the character of the isolate has not changed because of laboratory germination. Overall, there seems to be a need for standardizing future work so that the relative resistance of various microbes can be more readily compared. Unfortunately, the upper atmosphere remains mostly inaccessible to microbiologists due to the cost and difficulty associated with direct sampling, but future investigations can benefit greatly from laboratory simulations.

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