SYNERGISTIC EFFECTS OF LOW PRESSURE, LOW TEMPERATURE, AND CO_2 ATMOSPHERES INHIBIT THE GROWTH OF TERRESTRIAL BACTERIA UNDER SIMULATED MARTIAN CONDITIONS.

Andrew C. Schuerger[1] and Wayne L. Nicholson[2], [1]Dept. of Plant Pathology, University of Florida, Space Life Sciences Lab, Kennedy Space Center, FL 32899; acschuerger@ifas.ufl.edu; [2]Dept. of Microbiology, University of Florida, Space Life Sciences Lab, Kennedy Space Center, FL 32899; wln@ufl.edu.

Introduction: Mars spacecraft are assembled under strict conditions of sanitation and components are sterilized at various times during the payload integration process. Sterilizing procedures can include the use of dry-heat, wet-heat, gaseous steriliants, and/or chemical surface treatments depending on the spacecraft component and phase of assembly. Thus, the total bioburden at launch is constrained significantly and generally must be below 3×10^5 spores per vehicle for Category IV missions. Schuerger et al. [1,2] demonstrated that after spacecraft land on Mars, it is likely that sun-exposed surfaces can receive significant levels of UV irradiation to reduce the viable bioburden by up to 6 decades in as short a span as several tens of minutes under clear sky conditions (optical depth approximately 0.5). However, a significant portion of any landed vehicle will contain surfaces that are completely shielded from UV irradiation. The objective of the current study was to determine if common spacecraft contaminants shielded from solar UV irradiation can grow and replicate under the low pressure, low temperature, and high CO_2 atmospheres that are present at the surface of Mars.

Methods: Seven *Bacillus* spp. and six species of non-spore forming mesophilic bacteria were dispersed separately onto trypticase soy agar (TSA) by one of two methods and cultures immediately transferred to either a small bell-jar system or a Mars Simulation Chamber (MSC) (Figure 1). The bell-jar system was capable of maintaining pressures down to 7 mb, and the MSC system was capable of maintaining pressures down to 0.1 mb over extended periods. The bell-jar and MSC systems were configured with a CO_2 generation or injection system, respectively, to create pure CO_2 atmospheres. The bell-jar system could be maintained at temperatures down to 0 C, and the MSC system could be maintained down to –100 C.

Two assay procedures were used for all *Bacillus* spp., but only the vegetative-cell assay was used for the non-spore forming species. Vegetative cells (16-hr old cultures) of each species were streaked separately onto TSA in 2 or 3 quadrants, while flaming the transfer loop between quadrants. Endosproes were not present in the 16-hr-old cultures of all seven *Bacillus* spp. tested. Suspensions of separately prepared endospores of *Bacillus* spp. were then quantitatively calibrated to produce between 200 and 300 individual colonies per standard 15-cm petri dish of TSA. The vegetative cells or endopsores were then incubated in either the bell-jar or MSC systems for periods up to 7 days at various combinations of pressure, temeperature, and pure CO_2 atmospheres. Earth-controls were maintained at 30 C, 1013 mb pressure, and a standard O_2/N_2 atmosphere for all experiments.

Vegetative cells and endospores for all species were rated for robustness of growth using a simple rating system. Rating scale: 4 = large robust colonies > 5 mm in diameter; 3 = colonies 2-4 mm in diameter; 2 = colonies ≈ 1 mm in diameter; 1 = colonies ≈ 0.5 mm in diameter; 0.50 = colonies < 0.5 mm in diameter; 0.1 = smallest visually discernable colonies (pin-prick sized colonies at ≈ 0.1 mm in diameter); 0 = no growth. In addition, endospores were rated for colony

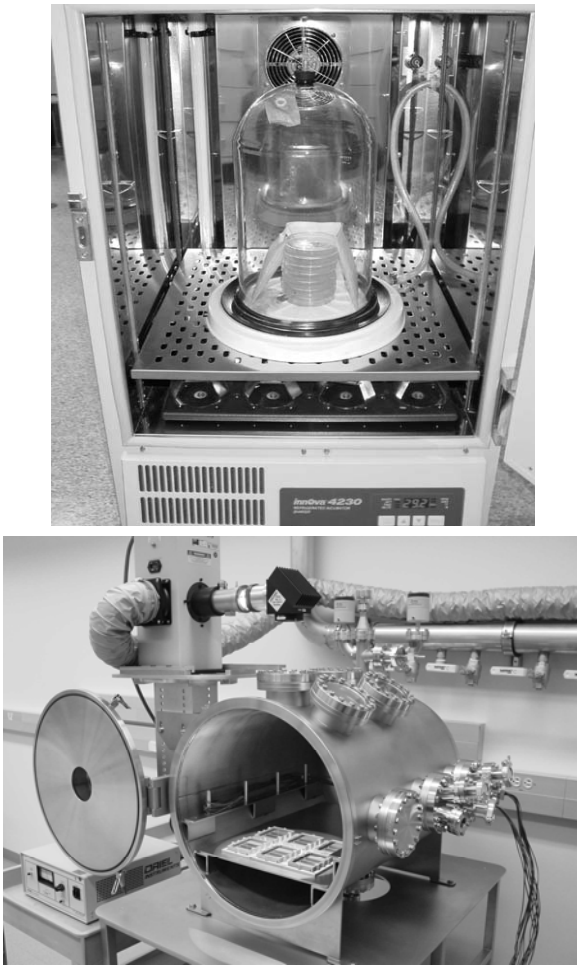

Figure 1. Top: The bell-jar system installed within a microbiological incubator. **Bottom:** The Mars Simulation Chamber (MSC) with the xenon-arc UV-VIS-NIR irradiation system installed.

numbers and colony diameters for all environmental treatments.

Results: The temperature minimum for vegetative cells after 48 hrs incubation was observed to be 15 C for all seven *Bacillus* spp. under an Earth-normal O_2/N_2 atmosphere, and 20 C for all seven *Bacillus* spp. under a pure CO_2 atmosphere. Thus, CO_2 and low temperature acted synergistically to raise the temperature minimum for all seven *Bacillus* spp. from 15 to 20 C for observable growth. Furthermore, the robustness of growth under pure CO_2 atmospheres was significantly lower than under O_2/N_2 atmospheres at all temperatures in which growth occurred in both gas compositions. For example, at 30 C, the average growth rating dropped from an average of 4 under O_2/N_2 atmospheres (rating 4 = robust growth with colonies between 5 and 8 mm in diameter) to approximately 1 under a CO_2 atmosphere (rating 1 = colonies ≈ 0.5 mm in diameter). The six non-spore forming mesophilic species (*Escherichia coli, Streptomyces coelicolor, Deinococcus radiodurans, Acinetobacter caloaceticus, Comamonas acidovorans, and Clavibacter michiganensis*) were tested only at 30 C.

Vegetative cells of all seven *Bacillus* spp. were then grown in pure CO_2 atmospheres under five pressures for 48 hrs at 30 C. The pressures tested were 1013 (Earth-normal control), 100, 50, 35, or 25 mb. Growth for six of the seven *Bacillus* spp. was observed down to 25 mb; only *B. megaterium* failed to exhibit evidence for positive growth at 25 mb. However, it must be emphasized that the growth at 25 mb for all six species that exhibited growth was at the lower limit of detection and did not always occur in each repetition of the experiment. Thus, although positive ratings were logged for several species at 25 mb and 30 C, the evidence for growth at 25 mb was at times equivocal. All seven *Bacillus* spp. grew at 35 mb and 30 C

In a third series of tests, vegetative cells of the seven *Bacillus* spp. were incubated for 48 hrs and 20 C at 15 or 25 mb in pure CO_2 atmospheres. In addition, bacteria were incubated on both TSA and TSA amended with glucose and nitrate (TSAGN). This assay was conducted to determine if lowering the temperature slightly (from 30 to 20 C) and providing glucose and nitrate (to enhance anaerobic metabolism) could extend the low-pressure thresholds of the vegetative cells of the seven *Bacillus* spp. Results indicated that all seven *Bacillus* spp. were not able to grow at 15 mb on either the TSA or TSAGN media, and that only *B. subtilis* 42HS-1 grew at 25 mb on TSAGN. The glucose and nitrate amendments increased growth only slightly at the low temperature, low pressure, and high CO_2 conditions used in these experiments.

In contrast to vegetative cells, endospores from all seven *Bacillus* spp. had significant difficulty in germinating and then growing at low pressures. A series of tests were conducted for 48 hrs at 30 C in which endospores from all seven *Bacillus* spp. were maintained under either O_2/N_2 or CO_2 atmospheres. In all tests, no endospores were observed to germinate and grow on TSA at 25 mb under either O_2/N_2 or CO_2 atmospheres. This is in contrast to the growth of vegetative cells under CO_2 atmospheres in which positive growth was observed in six of the seven *Bacillus* spp. down to 25 mb when cultures were grown at 30 C. At 35 mb under O_2/N_2 atmospheres, only endospores of *B. subtilis* HA-101 were observed to germinate and grow. In contrast, only endospores of *B. nealsonii* and *B. licheniformis* were observed to form observable colonies on TSA under CO_2 atmospheres at 35 mb. The observation of this dichotomy, in which different species grew only under CO_2 or O_2/N_2 atmospheres at 35 mb, was very consistent among the several repetitions of the experiment. Endospores from all seven *Bacillus* spp. were observed to germinate and grow at 50 mb and 30 C.

A series of tests were conducted with non-spore forming bacteria in order to determine if mesophilic non-spore forming species were also unable to grow at low pressures. The procedures were identical to those used for the vegetative cells of *Bacillus*, and bacteria were incubated 48 hrs under either O_2/N_2 or pure CO_2 atmospheres maintained at 30 C. Results indicated that only *E. coli* was capable of growth at 25 mb under CO_2. All other species (*S. coelicolor, D. radiodurans, A. caloaceticus, C. acidovorans,* and *C. michiganensis*) failed to grow at 25 mb under either O_2/N_2 or pure CO_2 atmospheres.

Conclusions: These results indicate that of the bacteria tested (13 species) all species had significant difficulties growing at pressures and temperatures that began to approach those found on the surface of Mars. No bacteria were found capable of growth at 15 mb, and most had difficulty growing at 25 mb under pure CO_2 atmospheres. Although this conclusion cannot be extended beyond these few species, the results do suggest that the microorganisms that remain viable on spacecraft surfaces after the 6-8 month transit time to Mars may not be readily capable of growth on the surface of Mars, even if exposed to a water and nutrient rich substrate. However, in order to broaden this conclusion into a more established paradigm for Mars, many additional tests are required with significantly greater species diversity and with many additional procedures for measuring growth and replication than were used here.

References: [1] Schuerger et al., (2003) *Icarus*, 165:253-276; [2] Schuerger et al., (2005) *Icarus*, (in preparation).