DIRECTED EVOLUTION OF *BACILLUS SUBTILIS* TOWARDS HYPOBARIC GROWTH.
Wayne L. Nicholson, Dept. of Microbiology and Cell Science, University of Florida, Space Life Sciences Lab, M6-1025, Room 201-B, Kennedy Space Center, FL 32899 USA. WLN@ufl.edu.

Introduction: Much astrobiology research is concerned with defining the environmental limits for life in the universe. Because Mars currently is the primary target for life detection missions, it is important to understand how terrestrial microbes might survive, proliferate, and evolve in martian environments. This issue is relevant in three distinct but related contexts: (i) testing panspermia hypotheses [1], (ii) mitigating the forward contamination of Mars [2], and (iii) understanding the molecular mechanisms leading to microbial growth in extreme extraterrestrial environments [3]. Prime candidates for Earth-to-Mars transfer include bacteria of the genus *Bacillus,* spores of which are significant contaminants of Mars-bound spacecraft [4]. It is thus relevant to assess the potential for such microbes to survive and proliferate in the martian environment.

The martian atmosphere poses a significant barrier to growth of terrestrial microbes, due to its low pressure (1-10 mbar; average 7 mbar) and anoxic (~95% CO_2) composition. In an earlier study [5] we showed that low pressures approaching those found on the surface of Mars exhibited an inhibitory effect on the germination and vegetative growth of several *Bacillus* spp. isolated from spacecraft or their assembly facilities. Even in an Earth-like 80%N_2/20%O_2 atmosphere, growth of *B. subtilis* cells was nearly completely inhibited at pressures below 35 mbar, well above the highest pressure on the martian surface [5]. The purpose of the present investigation was to use low pressure as a selective pressure to test the hypothesis that a terrestrial microorganism, *B. subtilis*, could evolve the ability for enhanced growth under hypobaric conditions approaching those of Mars.

Materials and Methods: Luria-Bertani (LB) medium was used throughout. Antibiotics used were spectinomycin (Spc, 100 μg/ml) and chloramphenicol (Cm, 5 μg/ml). Congenic *B. subtilis* wild-type strains WN624 (Spc^R) and WN628 (Cm^R) have been described previously [6] are were used as ancestral strains. Strains were propagated in LB liquid medium containing the appropriate selective antibiotics at 27˚C with shaking in Earth atmosphere at a pressure of 1013 mbar (1 atm; WN628) or at 50 mbar (WN624). At 24-hour (~6.6 generation) intervals, culture optical densities at 660 nm (OD_{660}) were recorded, cultures diluted 1:100 into fresh selective medium, and propagation continued. After 1,000 generations of propagation, single-colony isolates were obtained from each culture and designated WN1105 (evolved at 1013 mbar) and WN1106 (evolved at 50 mbar), respectively.

Relative fitnesses of evolved vs. ancestral strains were determined by competition experiments. Equal numbers of cells of each strain were inoculated into LB medium with no antibiotics and cultivated as described above at 1013 mbar or 50 mbar for 7-8 days (~50 generations). At 24-hour intervals, samples were removed from cultures and viable counts of each strain were determined by serial tenfold dilution and plate counts on LB containing the appropriate antibiotic. The proportion of each strain in the mixed cultures were plotted vs. generation to determine competitive fitness.

Results and Discussion: Propagation of both strains WN628 or WN624 at 1013 or 50 mbar for 1,000 generations resulted in an overall increase in 24-hour OD_{660} values. Increases were seen to occur in a stepwise fashion, suggesting that evolution of the strains was accomplished through a sequence of mutational events and population sweeps. Both evolved strains WN1105 and WN1106 had gained fitness relative to their wild-type ancestors when competitions were performed at the original pressure at which the respective strains had evolved. As might be expected, strain WN1106 was more fit at 50 mbar than WN1105, and WN1105 was more fit than WN1106 at 1013 mbar. Interestingly, strain WN1105 was less fit than the ancestor at 50 mbar, whereas WN1106 showed the same fitness at its ancestral strain at 1013 mbar. Transcription microarrays are in progress to understand (i) differences in gene expression in cells at 1013 vs. 50 mbar and (ii) differences in gene expression of ancestral vs. evolved strains at 50 mbar.

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