SEARCH FOR EXTANT LIFE ON MARS: USE OF NANOSIMS TO CHARACTERIZE THE LOWER LIMITS OF MICROBIAL METABOLISM AT LOW TEMPERATURES FOR THE BACTERIUM, *SERRATIA LIQUEFACIENS*. A. C. Schuerger[1], P. Schwendner[1], and A. N. Nguyen[2], [1]University of Florida, 505 Odyssey Way, Exploration Park, Merritt Island, FL 32953, email: schuerg@ufl.edu; petra.schwendner@ufl.edu; [2] Jacobs, NASA JSC, Houston TX, email: lan-anh.n.nguyen@nasa.gov.

Introduction: The search for life on Mars is a primary goal of astrobiology. In an effort to help constrain potential habitable zones (HZ) on the Martian surface, we conducted a series of experiments to examine the lower limits of metabolism and growth for *Serratia liquefaciens* at subzero temperatures. The bacterium, *S. liquefaciens*, was selected for these experiments because it can metabolize organics and grow under simulated Martian conditions of low-pressure (0.7 kPa), low-temperature (0°C) and CO_2-enriched hypoxic atmospheres [1,2,3,4]; henceforth called *low-PTA* conditions.

The goal of this research was to characterize the lower limits of metabolism and growth at sub-zero temperatures with *S. liquefaciens* as a prelude for conducting more complex simulations under Mars low-PTA conditions at even lower pressures and temperatures than have been completed heretofore.

Nano-scale secondary ion mass spectrometry (NanoSIMS) is a high spatial resolution method creating nanoscale maps of isotopic composition and estimating isotopic ratios. These features can be used to probe the metabolic states of cells when the growth media are spiked with stable isotopic compounds. In the present study, NanoSIMS was used to characterize the metabolic threshold in *Serratia liquefaciens* grown at temperatures at and below 0°C.

Methods: *Microbial protocols.* Cells of *S. liquefaciens* ATCC 27592 were grown in Spizizen medium supplemented with three stable isotopes: 20 mM ^{13}C-glucose (^{13}C, 99%), $(^{15}NH_4)_2SO_4$ ($^{15}N_2$, 99%), and $H_2{}^{18}O$ (^{18}O, 97%); completely substituting their unlabeled counterparts. The cells were incubated in 96-well plates at 0, –1.5, –3, –5, –10, or –15°C for 4 wks for NanoSIMS analyses and 8 wks for optical density growth curves. Growth rates and survivability were determined by optical density measurements and by applying the Most Probable Number (MPN) assays after 10-fold serial dilutions [5]. For NanoSIMS analysis, cells were harvested, fixed in 2 % glutaraldehyde in 1x phosphate buffered saline (PBS), applied to a filter, washed in PBS, dehydrated in a graded ethanol series, critical-point dried, mounted on SEM stubs, and coated with gold-palladium. The cells were imaged in an Hitachi Su5000 scanning electron microscope to map the sample prior to viewing the cells in the NanoSIMS (located at NASA JSC, TX).

NanoSIMS protocols. The NanoSIMS Cameca 50L instrument was used to conduct isotopic analyses of the cells. An ~1.5 pA Cs^+ primary ion beam was rastered over 20x20 μm fields of view for 20 layers. The isotopes ^{12}C, ^{13}C, ^{16}O, ^{18}O, $^{12}C^{14}N$, $^{12}C^{15}N$, and ^{32}S were simultaneously analyzed as negative secondary ions in electron multipliers. The ion counts for each isotope were summed over all layers and isotopic ratios were deduced for individually defined cells. The unlabeled cells incubated at 0°C were measured prior to analysis of each labeled treatment. The isotopic ratios of the labeled cells were normalized to those of the unlabeled cells. Measurements of San Carlos olivine grains and kerogen were conducted prior to analysis of the cells to tune the instrument and ensure good measurement reproducibility. Isotopic ratios are expressed as delta values; e.g., $\delta^{13}C/^{12}C$ (‰) = $[(^{13}C/^{12}C)_{labeled}/(^{13}C/^{12}C)_{unlabeled}-1] \times 1000$.

Statistics: Delta (δ) values (‰) for ^{13}C, ^{15}N, and ^{18}O uptake were log10 transformed to induce homogeneity of treatment variances and then analyzed with ANOVA and protected least-squares mean separation tests ($P \leq 0.05$). The data in Table 1 are presented as untransformed means for the $\delta\ ^{13}C/^{12}C$ (‰) concentrations in the imaged cells. Only the $\delta\ ^{13}C/^{12}C$ data are presented here; $^{18}0/^{16}0$ and $^{15}N/^{14}N$ data will be presented at the conference.

Results: *Microbial growth results.* After 4 weeks, visible growth was observed in cultures for only the samples incubated at 0°C. However, after 8 weeks, the medium turned turbid in samples incubated at –1.5 and –3°C indicating growth (albeit at slow rates) for the sub-zero temperatures indicated. In contrast, cell densities in cultures incubated at –5, –10, or –15°C decreased by at least ½ order of magnitude over 8 weeks.

NanoSIMS results. Individual cells in the NanoSIMS images (total of 240 cells) were outlined as individual regions of interest (ROIs) and the isotopic ratios $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{18}O/^{16}O$ were determined. Augmentation of the growth media with ^{13}C-glucose dramatically increased the cell concentration of ^{13}C ions for cells of *S. liquefaciens* grown at 0°C; supporting previous studies that showed active metabolism and growth for *S. liquefaciens* under simulated Mars low-PTA conditions [1,2,3,4]. However, as the temperature was lowered, metabolism was greatly reduced at –1.5

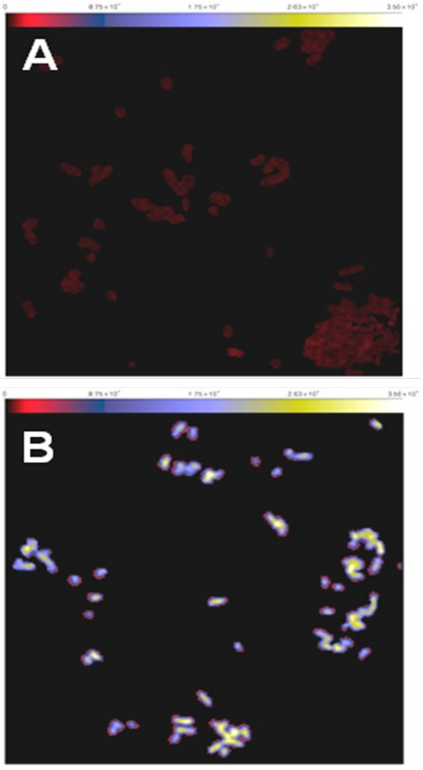
and –3; almost halted at –5°C, and shut-down completely at –10 and –15°C (Table 1). Cells incubated at –10 and –15°C were not significantly different from the unlabelled cells incubated at 0°C.

Table 1. δ ^{13}C (‰) means for *S. liquefaciens* cells incubated at subzero temperatures.

Temperature/^{13}C labeling	δ^{13}C (‰) [1]
0°C/unlabeled cells	8.3 e
0°C/labeled cells	17,178 a
–1.5°C/labeled cells	3673 b
–3°C/labeled cells	1756 c
–5°C/labeled cells	400 d
–10°C/labeled cells	-22.4 e
–15°C/labeled cells	21.2 e

[1] Values designated with different letters were significantly divergent from the unlabelled cells grown at 0°C. Delta (δ) ^{13}C (‰) values were analyzed with ANOVA and protected least-squares mean separation tests as described in the text ($P \leq 0.001$; n varied between 24 and 67 cells per treatment.

Figure 1 shows the range of NanoSIMS images acquired during these experiments. Figure 1A (top) shows cells of *S. liquefaciens* incubated on standard media (i.e., unlabeled) grown at 0°C. Figure 1B (bottom) shows cells grown on ^{13}C-glucose labeled media incubated at 0°C. These images exhibit the lower and upper extremes in the ^{13}C/^{12}C images. All other treatments exhibited δ ^{13}C (‰) values between these two extremes (see Table 1).



Discussion: NanoSIMS protocols and resultant datasets are similar to those described for other bacteria incubated under diverse growth-limiting conditions [6,7]. The NanoSIMS approach provides unequivocal evidence on the limits for metabolic activity for microorganisms exposed to extreme conditions generally considered inimical for metabolism and growth.

Here, cells of *S. liquefaciens* incubated with ^{13}C-glucose were metabolically inhibited by the slight reduction in temperature from 0 to –1.5°C. The high δ^{13}C (‰) mean value of 17,178 for labeled cells grown 28 d at 0°C suggests that >99.99% of the organic-C in the cells were converted to labeled ^{13}C macromolecules over the course of 28 d. The δ^{13}C (‰) value decreased by ~80% when cells were incubated at –1.5°C, by 90% at –3°C, and by 98% at –5°C. The results for –10°C and –15°C cultures suggest that cells of *S. liquefaciens* were not metabolically active regarding ^{13}C-glucose uptake at these temperatures. It is unknown if other labeled ^{13}C organics might be useable by *S. liquefaciens* cells at ≤ –5°C

On Mars, liquid water can be maintained only within a very narrow window close to the triple point of water (i.e., 0.01°C at 6.1 mbar) [8]. *Serratia liquefaciens* is a hypopiezotolerant bacterium capable of metabolism and growth under simulated Mars low-PTA condition [1,2,3,4]. However, the results herein suggest that even for the low-pressure tolerant *S. liquefaciens* bacterium, the range of tolerance to low-temperatures below 0°C is very narrow. Thus, it seems unlikely that *S. liquefaciens* would be able to grow on the surface of Mars (with a global average temperature of –61°C), even if mild salt brines were able to slightly depress the temperatures to no lower than –3°C, and abundant organics and water were present; all very unlikely on the surface on modern-day Mars.

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References: [1] Schuerger A. C. et al. (2013) *Astrobiology*, 13(2), 115-131. [2] Schuerger A. C. and Nicholson W. L. (2016) *Astrobiology*, 16(2), 964-976. [3] Fajardo-Cavazos P. et al. (2018) *Scientific Reports*, 8:14938, doi:10.1038/s41598-018-33140-4. [4] Schwendner P. and Schuerger A. C. (2018) *Scientific Reports*, 8:15721, doi:10.1038/s41598-018-33856-3. [5] Mancinelli R. L. and Klovstad M. (2000) *Planet. Space Sci.*, 48, 1093-1097. [6] Musat N. et al., (2014) *Systematic Appl. Microbiol.*, 37, 267-276. [7] Li T. et al. (2007) *Environ. Microbiol.*, 10(3), 580-588. [8] Haberele R. M. et al. (2001) *J. Geophys. Res.*, 106, 23,317-23,326.