

# *Bacillus Pumilus* SAFR-032L: A Model for Planetary Protection Research

by George E. Fox

**ABSTRACT**—In order to prevent forward contamination of Mars, it is necessary to minimize the bio-burden on spacecraft that will contact the Martian surface. *Bacillus pumilus* strains whose spores are unusually resistant to ultraviolet light and other means of sterilization have been consistently found in the clean rooms used in space craft assembly as well as on space craft. In order to better understand the origin of these organisms and to develop effective means for eliminating them, the complete genome of *Bacillus pumilus* SAFR-032 is being sequenced in collaboration with the Baylor College of Medicine Human Genome Sequencing Center (HGSC). The sequence is in the finishing stage, and annotation is nearly complete. Comparisons of the genes involved in the sporulation process and UV repair are being intercompared in detail to identify genomic features that might be responsible for the unusual resistances that this organism exhibits.



A PRIMARY OBJECTIVE OF THE SPACE SCIENCES IS TO SEARCH for evidence of living systems in the universe. The most tractable target for such efforts has been and for the near future will continue to be the planet Mars. Having ratified the 1967 Outer Space Treaty, the United States is obliged to avoid harmful contamination of celestial bodies that might harbor life. Spacecraft that land on Mars but are not equipped with life-detection experiments must minimize the bio-burden they bring to the planet. Thus, the components are subjected to rigorous cleaning and must be assembled in a Class 100,000 clean room or better. If the mission contains life-detection experiments, a sterilization process must be applied as good as or better than that applied to the landers used in the 1967 Viking missions.

In preparing that protocol, researchers argued that the organisms most likely to be resistant to sterilization would be endospore-forming bacteria of the genera *Bacillus* and *Clostridium*. Hence, a spore assay was developed to evaluate the effectiveness of the sterilization process using *Bacillus subtilis* as the model organism.

Given this background, modern planetary protection research focuses on two issues: (1) development of cleaning and sterilization technologies and better methods of evaluating their success and (2) the assessment of which terrestrial microorganisms are most likely to survive cleaning and sterilization and hence possibly confound tests for life done on the Martian surface or contaminate returned samples.

Spacecraft are assembled in clean rooms which employ sterilants including vapor-phase hydrogen peroxide (VHP), a

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strong oxidizing agent, and UV, in addition to physical containment to obtain and maintain the necessary pristine surfaces. While conducting a microbial census of NASA spacecraft assembly facilities, Venkateswaran and his colleagues discovered that strict contamination control measures did not provide an absolute barrier to microbial contamination but, instead, established a series of selective “bottlenecks” which are sufficient to prevent the penetration or survival of all but the hardiest microbes into the interior assembly area.<sup>1</sup> The predominant isolates repeatedly found to have penetrated deepest within the cleanest parts of the spacecraft assembly facilities were spore-forming bacteria, especially strains of *Bacillus pumilus*, *B. nealsonii*,<sup>2,3</sup> and a closely related grouping recently named *B. odysseyi* because it was first isolated from the surface of the Mars Odyssey spacecraft.<sup>4</sup> *B. pumilus* strains have also regularly been isolated from space craft including hardware from the International Space Station (ISS). In comparison with the model organism *B. subtilis*, which is used in the standard spore assay, *B. odysseyi* spores had survival times that were 3 times, 10 times and 6 times longer when exposed to UV, gamma radiation, and hydrogen peroxide. The most resistant strain isolated to date, *B. pumilus* SAFR-032 (Space Craft Assembly Facility Resistant Isolate 32) has at least a 10-fold increase in its resistance to Mars UV radiation conditions

than the standard *B. subtilis*.

In view of these findings, it is clear that assays for cleanliness and survival potential will be more meaningful if they are based on the organisms that are likely to be problematic such as *B. pumilus* and *B. oddysseyi*. Of the various resistant *Bacillus* strains that were isolated, the SAFR-032 isolate is the most resistant and has been selected for two key follow-up studies. First, a flight experiment was proposed and has been selected for a study of the effect of radiation resistance on this organism. This experiment will be carried out aboard the ISS using the European Technology Exposure Platform and Experiment Facility (EXPOSE). Second, the complete sequence of the genome of SAFR-032 is currently being determined by investigators at the Baylor College of Medicine Human Genome Sequencing Center (HGSC) with funding from the National Science Foundation (NSF).

### Methodology

The SAFR-032 genome is essentially complete, and the HGSC group is currently conducting finishing studies in order to finalize the assembly of several very large reliable contigs. In addition, partial data were obtained for a second strain, F036B. A high-quality manual annotation is in progress using the HGSC-developed CONAN interface. Possible open reading frames are initially predicted by Glimmer<sup>5</sup> and GeneMarkS<sup>6</sup> and then automatically examined for similarity to known COGS, protein domains and other annotated genes (i.e., pre-run BLAST searches). The annotator looks at all the data and in some cases literature references before assigning a name and if possible a likely function for the open reading frame.<sup>7</sup> Each gene is annotated separately by two annotators and the resulting annotations compared automatically. Differences are subsequently resolved by conference between the annotators with input from the group as a whole when needed.

The initial analysis will identify genes that are shared or not shared with *Bacillus subtilis*<sup>8</sup> and other *Bacillus* genomic sequences. Of special interest will be genes known to be involved in spore resistance<sup>9-12</sup>, DNA repair,<sup>13-15</sup> and sporulation in general.<sup>16,17</sup> Examination of shared genes will allow us to assess whether regulatory signals, e.g., promoter sequences for various sigma factors, are changed or not. In particular, genes known to be associated with particular sigma factors can be used to define recognition sites in SAFR-032. With this knowledge in place, genes that are uniquely present or absent in SAFR-032 will be broken into two groups, those associated with known sporulation genes and regulons and those not so associated. The leader regions of unique genes not associated with known sporulation operons will be further examined to see if they are likely to be transcribed by any of the sigma factors associated with sporulation.<sup>18</sup> A second analysis will be a detailed comparison of the SAFR-032 gene organization with the well established operon structure of *B. subtilis*<sup>19</sup> to identify possible regulatory differences. The most promising changes here will likely be operons whose structure is uniquely changed in SAFR-032 relative to the other known *Bacillus* genomes, all lacking resistant spores.

### Results

As a first step toward understanding the biology of these unique strains, the whole genome sequence of one isolate, *B. pumilus* SAFR-032, was determined. The *B. pumilus* SAFR-032 genome is 3.62 MB in size with approximately 3950 genes. Sequence alignment and subsequent construction of a tree of phylogenetic relationship for multiple housekeeping genes revealed that among published complete genomes, SAFR-032 is most closely related to *B. subtilis* and *B. licheniformis*, thereby rendering these organisms of greatest relevance for comparison. Since the sporulation machinery of *B. subtilis* has been extensively studied, this is an especially favorable outcome. A similar analysis of the F036B strain of *B. pumilus* revealed that it is much closer to SAFR-032 than the latter is to *B. subtilis* or *B. licheniformis*. A comparison of gene order between SAFR-032 and *B. licheniformis* revealed substantial co-linearity. This is important because one can scan local clusters for gene additions or losses that may correlate with changes in regulation.

### Discussion

Despite the substantial similarity to *B. licheniformis* and *B. subtilis*, there are, nevertheless, many coding regions in SAFR-032 with no obvious homolog in these other organisms. Genes known to be involved in the sporulation processes, including regulation, spore protection, and germination, were compared with their homologs in *B. subtilis* and *B. licheniformis* in order to identify unusual absences and changes in gene order. The largest number of such changes are associated with genes involved in the generation of the spore coat protein which has likely been redesigned in *B. pumilus* relative to its sister species. Genes encoding the small acid soluble proteins, which are not only crucial for spore DNA protection but also known to be highly conserved within and across species, have interesting sequence variation, as compared to those of other *Bacillus* strains. Further, detailed comparative analysis revealed that several proteins annotated as hypothetical in *B. subtilis/B. licheniformis*, could actually be classified as functional genes (such as families of transporters, transcriptional regulator proteins etc.), genes unique to *B. pumilus*. Researchers found also that gene clusters encoding the polyketide pathway have substantial sequence variation from those of other *Bacillus* strains.

### Conclusions

Results obtained here will in the short term generate multiple candidate genomic features that may separately or in combination be responsible for the unusual resistance associated with *B. pumilus* spores. In the immediate future it will be of interest to explore these alternative possibilities by comparing gene expression patterns in resistant and non-resistant strains. Funding for this purpose will be sought from the National Science Foundation and other federal agencies. In the longer view, the processes by which non-resistant strains readily evolve resistance to UV will be a useful model system for studying evolution.

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## Funding and Proposals

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- Fox, G. E. “Comparative Genome Analysis and the Resistance Properties of Various *Bacillus* Species,” NASA-Planetary Protection Program: Feb. 1, 2005–Jan. 31, 2008. UH Total Costs: \$226,723. (Not funded.)