MOLECULAR ANALYSIS OF SPACEFLIGHT EFFECTS ON SEVERAL SPECIES OF MICROORGANISMS
B.H. Pyle¹, T.L. Goins¹, K.S. Lim¹, A.M. Smith¹, S.C. Broadaway¹, T.A. Voeikova², V.K. Ilyin³
¹ Montana State Univ., Microbiology Dept., 109 Lewis Hall, Bozeman, MT 59717, USA.
² GosNIIGenetika, 1 Dorozhnyi proezd, Moscow 117545, Russia.
³ Institute for Biomedical Problems, Khoroshevskoye Shosse 76A, Moscow 123007, Russia.

ABSTRACT
Bacterial cultures were flown for 12 days on the Russian Foton-M3 spacecraft to detect genetic changes in response to spaceflight conditions. Flight experiment (FE) and asynchronous ground control (AGC) cultures of Streptomyces lividans (pIJ702) were screened for putative mutations in the tyr gene required for melanin synthesis. In flight samples, plasmid expression in FE clones was 3-fold greater than that for AGC cultures. Gene sequencing detected G-C base-pair substitutions in the tyr gene in both FE and AGC cultures. Streptomyces coelicolor strain Ac-236 with several auxotrophic marker genes was screened for spontaneous revertants and the arginine and proline genes were amplified for sequencing to characterize the genetic changes resulting in prototrophy. Ribosomal DNA was extracted from FE and AGC cultures of Pseudomonas aeruginosa, Bacillus pumilus, Escherichia coli and Lactobacillus casei and analyzed for mutations. No genetic changes were detected in these species. The results suggest that there is limited potential for mutation when cultures and cells are subjected to spacecraft flight conditions in short-duration low earth orbit.

INTRODUCTION
Microorganisms selected for three studies were flown for 12 days on the Russian Foton-M3 spacecraft and were analyzed to detect genetic changes in response to spaceflight conditions.

Flight experiment (FE) and asynchronous ground control (AGC) cultures of Streptomyces lividans containing plasmid pIJ702 (Figure 1) [Jones & Hopwood, 1986; Gusek & Kinsella, 1992] were screened for putative mutations in the tyr gene required for melanin synthesis. The plasmid contains a thiostrepton resistance determinant (tsr) and the tyrosinase (melanin) gene (mel) from S. antibioticus. The tyr sequence was amplified by PCR and the product sequenced to characterize mutation(s) resulting in loss of function to determine if space flight affected the type and or frequency of mutation(s).

Conjugation and genetic recombination between two parental Streptomyces coelicolor strains was examined in FE and AGC. The “donor” A3(2) strain contained the conjugal plasmid SCPI that enabled the transfer of DNA to the “recipient” parental strain that lacked the SCPI plasmid. Reversion to prototrophy is a normal occurrence, and several arginine and proline revertants were isolated and the genes sequenced to characterize the genetic change(s) resulting in arginine and proline prototrophy. Recombinant progeny were analyzed to evaluate space flight effects on plasmid SCPI mobilization capabilities and recombination events.

Figure 1. Plasmid pIJ702, showing locations of tsr and mel genes and selected restriction enzyme sites [Jones & Hopwood, 1986].

Nucleic acids extracted from FE and AGC cultures of P. aeruginosa, B. pumilus, E. coli and L. casei were analyzed for frequency and type of mutation in variable regions of the rDNA. These mutations may occur with no lethal effects on the organism, so the mutation may be amplified through replication. Double denaturing gradient gel electrophoresis (DGGE) and sequence analysis were used to detect mutations.

MATERIALS AND METHODS
Bacterial cultures were flown for 12 days on the Russian Foton-M3 spacecraft (September, 2007) to detect genetic changes in response to spaceflight conditions. Flight experiment (FE) and asynchronous ground control (AGC) cultures of Streptomyces lividans (pIJ702) were held at 30°C on solid media at 1 atmosphere, and the spacecraft was returned by aircraft to the investigators in Moscow within 14 hours of landing in Northern Kazakhstan. Methods of analysis were as follows.

S. lividans Plasmid Expression
Spore Collection. Working under a laminar flow hood, each culture plate (FE and AGC) was flooded with 9 ml of sterile water and the surface of the culture scraped to

* Correspondence to: Barry H. Pyle
Microbiology Department
Montana State University
Bozeman, MT, 59717
Email: barryp@montana.edu
Phone: 406-994-3041; Fax: 406-994-4926
release the spores and filamentous growth. The spore suspension was transferred to a tube and vortexed vigorously, before filtering through non-absorbent glass wool introduced as a plug in an autoclaved standard 10ml glass pipette by gravity filtering into another sterile centrifuge tube to collect spores released from the filaments. The collected suspension was centrifuged for 10 minutes at 1000g to pellet spores; the tube was then drained and resuspended with residual fluid and sterile 20% glycerol added and agitated briefly before freezing at -20°C for storage.

**Growth Media.** ISP agar, ISP agar with thiostrepton, Tryptic Soy Starch Agar (TSSA) and Tryptic Soy Starch Agar with thiostrepton (TSSA+Ts) were used. TSSA was prepared by adding 7.5g tryptic soy broth, 5g potato starch, 10g agar, 10µl copper (II) sulphate solution (2.5%, final concentration 5ng/ml) and 150mg of L-tyrosine to 500ml of water, and autoclaving for 20 minutes at 121°C. The sterile media were tempered in a 55°C water bath before pouring plates. For ISP+Ts and TSSA+Ts, 500µl of antibiotic (50mg of thiostrepton in 1 ml DMSO) was added before pouring plates.

**Plasmid Expression.** To determine plasmid expression, 100µl of appropriate dilution was spread plated to both TSSA and TSSA+Ts plates. Plates were incubated at 30°C for three days. After incubation, colonies were counted and compared to determine plasmid expression rate.

**Variant Isolation.** 100µl of an appropriate dilution was spread plated onto TSSA+Ts plates. The colonies recovered on included only those spores that retained the plasmid since they were thiostrepton resistant. Clones that retained the variant mel pigment production were plated to another new plate to obtain sufficient growth for DNA isolation.

**Replica Plating.** Appropriate dilutions of spore suspension that would yield 10 to 30 colonies were spread plated on ISP media without thiostrepton and incubated at 30°C for 3 days. Colonies on ISP agar were replicated onto ISP agar without thiostrepton and onto ISP agar with thiostrepton (for thiostrepton sensitivity). After replication, plates were incubated for 2 days at 30°C. Colonies on replica plates were counted to determine thiostrepton sensitivity and pigment production. Replica plating from ISP agar to all four media (ISP, ISP+Ts, TSSA, TSSA+Ts) was also carried out [Smith et al., 2009].

**DNA Isolation and Analysis.** Approximately 100µl of cell spore suspension was transferred to a clean mortar with 750µl of Carlson Lysis Buffer [Carlson et al., 1991] and a pinch of grinding sand. The mixture was ground thoroughly, transferred to a 1.5ml microfuge tube and heated at 70°C for 30 to 40 minutes, inverting the tube every 10 minutes. The sample was centrifuged at room temperature, and the supernatant transferred to a new tube. Then, 750µl of 24:1 chloroform to isoamyl alcohol was added and mixed well by inversion. The sample was centrifuged and the upper aqueous phase transferred to a clean tube, and an equal volume of isopropanol added and mixed by gentle inversion to sediment DNA, then centrifuged and supernatant removed. The pellet was washed with ice cold 70% EtOH and then dried for about 3 minutes. DNA was suspended in 80 µl of TE buffer (10mM Tris, 1mM EDTA; pH 8.0) and treated with 2µl of RNase Plus at 37°C for 30 min. All centrifugation was done at 10,000g for 10 min.

For DNA analysis, the gene sequence in the variant clones (loss of pigmented phenotype) was amplified by polymerase chain reaction using the primers Tyr F forward (AACTGCATGTGATGCGCAACGC) and Tyr R reverse (GTGTGTTCCACGGCCTTCAT) primers to yield a 945 bp product. Gel electrophoresis was carried out on PCR products for size determination.

**Sequencing:** PCR products were purified using a QIAquick PCR purification kit (Qiagen) protocol. Purified PCR products were then diluted to an appropriate concentration before being sent to Functional Biosciences, Inc. (Wisconsin) for sequencing.

**S. coelicolor Recombination**

The prototrophs were cultured on selective medium to verify phenotype and the DNA extracted for amplification and sequencing to characterize the mutations resulting in reversion to prototrophy. The entire contents (growth and agar medium) of each selective agar plate was transferred to 100 ml water, autoclaved until agar melted, filtered and growth collected in a microcentrifuge tube containing microbeads. Carlson lysis buffer (1 ml containing 2 µl/ml β-mercaptoethanol) was added followed by bead-beating for 20 seconds. The extraction procedure was essentially above, except that DNA was suspended in water rather than TE buffer and not treated with RNase. DNA concentration was determined with a NanoDrop spectrophotometer followed by PCR for arg, phe and pro gene sequences.

**Microbial Growth and Mutation**

DNA was extracted as described above. The nine variable regions of the 16S rRNA operon were amplified using specific primer pairs based on the *E. coli* rrmD sequence. Primers were verified as appropriate DNA targets in all four organisms. Analysis of flight and ground samples included identification of putative mutants by double DGGE, the elution of bands of interest, and PCR amplification for sequence analysis using the Vector NTI program, FinchTV and NCBI database.

**RESULTS**

**S. lividans Plasmid Expression.**

The rate of plasmid expression in flight samples (94% of 860 colonies) was about 3-fold greater than that in ground controls (30% out of 437) when cultures were grown on ISP agar (Figure 2). Ground cultures on ISP media also had a lower expression rate than cultures on minimum
media (80% of 1052). The results for screening thiostrepton resistant non-pigmented (tsr+ mel-) variants showed that there were slightly more variants in the ground samples (0.07%, 4 out of 5807) than the flight samples (0.03%, 1 out of 3347).

**Figure 2:** Mean number of colonies recovered from non-selective (no thiostrepton) and selective growth media.

**Figure 3.** Plasmid expression (%) determined from replica plating.

**Replica Plating.** The wild type (mel+ tsr+) was observed at the highest frequencies in both flight and ground samples (Figure 3). The mel- tsr+ phenotype was not isolated. The flight samples showed slightly greater expression of the wild type characteristics than the ground samples. The phenotypes mel+ tsr- and mel- tsr- were observed at a higher frequency in ground clones over flight clones (Figure 3). Colonies replicated onto media with thiostrepton grew slower than colonies replicated onto ISP media without thiostrepton.

Thiostrepton-sensitive non-pigmented mel- tsr- variants were also detected by replica plating; among 384 ground colonies there were 86 variants (22%) compared with 58 variants among 451 flight colonies (13%). Based on the numbers of tsr+ non-pigmented colonies that were recovered, there was a similar rate of loss of mel gene expression for flight and ground samples [4/5807 (0.07%) vs. 1/3347 (0.03%)]. The frequency of tsr+ mel- variants was similar in AGC and FE clones (7x10^-4 vs. 3x10^-4).

**Molecular Analysis.** Gel electrophoresis of amplified DNA (Figure 4) indicated that spaceflight did not induce major deletion or insertion mutations in DNA of mutants, since they had the same size of DNA fragments from the ground wild-type and ground variants, when using the primers that were designed to amplify DNA fragments of interest. Further analysis suggested that ground isolates can survive without the target genes because no target DNA was amplified in one of the Ground isolates (Figure 5).

**Figure 4:** Agarose gel showing mel DNA fragments for a range of variants from flight and ground clones.

**Figure 5.** Agarose gel showing mel DNA fragments for mel tsr variants from three ground and flight clones.

Sequencing showed G-C substitutions in the mel gene of isolates from both flight and ground cultures (Figure 6). Substitutions occurred at different loci on the plasmid to produce a non-functional gene. Amplified DNA fragments with these base-pair substitutions showed similar migration of PCR products on agarose gels (Figures 4 and 5).

**S. coelicolor Recombination**

Arginine (Figure 7) and proline (Fig. 8) prototrophs from FE and AGC cultures plated on the selective media yielded a variety of pigmented isolates. PCR amplification of DNA from the two parental strains SC-236 and SC-3110 demonstrated the detection of arginine, phenylalanine and proline gene sequences (Figure 9).

**Microbial Growth and Mutation.** Fig. 10 shows that while PCR products from different species amplified by the same primer pair migrated similarly on an agarose gel, they migrated at different rates in DGGE because of their differing guanine/cytosine ratios. The DGGE double band for 8gc/691 appeared consistently regardless of template. No mutations were detected between and of the FE and AGC culture pairs examined.

Agarose and DGGE results for *E. coli* (Figure 11) showed there were no differences between flight and ground samples in three amplicons.
Figure 6: Partial gene sequences amplified using Mel4F/Mel6R, showing nucleotides (in bold) that differ from the wildtype.

Figure 7. Arginine (Arg⁺) prototrophs from FE (Flight) and AGC (Ground) cultures of S. coelicolor.

Figure 8. Proline (Pro⁺) prototrophs from FE (Flight) and AGC (Ground) cultures of S. coelicolor.
DISCUSSION

*S. lividans* pIJ702 gave lower plasmid expression rates in ground vs. flight cultures. Non-expressing clones either had mutation(s) in the *tsr* or *mel* genes or had lost the plasmid. The higher proportion of clones that expressed the plasmid from flight samples suggests that spaceflight factors may have selected for cells that retained the functional plasmid. This may be related to enhanced survival of plasmid expressing clones in response to stress [Dyson & Schrempf, 1987] because the plasmid was expressed by flight culture spores that were analyzed by subculture after samples were returned to the laboratory.

Prototrophic revertants of *S. coelicolor* were isolated, and genes for arginine, phenylalanine and proline synthesis were amplified from the parent strains.

Differences in *rnnD* sequences from *E. coli* and *B. pumilus* were demonstrated by DGGE. There was, however, no evidence for mutations in the variable *rnnD* sequences following growth of *E. coli* in flight cultures or in ground controls.

Based on these results, there was no evidence of spaceflight-related genetic changes in the bacteria studied. This may be attributed to the relatively low radiation dose received by the microorganisms during the Foton-M3 flight. The radiation dose was reported to be 200-300 mrad (2-3 mGy) during the 12 days of orbit, which is similar to that recorded in previous Foton flights. It has been estimated [Todd, 2004] that point mutations in yeast would occur at $3 \times 10^{-8}$ per locus after 100 days in low earth orbit, or ca. $10^{-6}$ after 10 days. Since Foton-M3 was in orbit for 12 days, a mutation rate of ca. $10^{-4}$ in *S. lividans* is relatively high in comparison with results for yeast. However, rates for *S. lividans* flight and ground
samples were similar and reflect the inherent genetic variability of this species [Dyson & Schrempf, 1987].

Another factor that may have limited spaceflight effects on genetic variability was growth of the bacteria on solid media rather than in liquid culture. Research on effects of spaceflight factors on microorganisms suggests that effects of microgravity on gene expression are related to lowering shear stress and/or convection-diffusion rates [Wilson et al., 2007; Brown et al., 2002]. These factors are observed mainly in liquid culture rather than on solid agar surfaces.

There was no evidence of mutations in *S. lividans* 66 pIJ702 in a similar experiment on Foton-M2 [Tabakov et al., 2006], although growth temperatures for the organism were not optimum in that case. However, it was found that slower growth at a lower temperature without complete differentiation produced a lower proportion of clones that expressed the pIJ702 plasmid from both flight and ground samples. This suggests a different selective effect for slow-growing and/or incompletely differentiated cultures.

**CONCLUSIONS**

The results of these investigations may lead to optimism in terms of human health risks related to spaceflight, particularly with respect to genetic effects on both microorganisms and higher organisms including humans. However, it is likely that growth of bacteria in liquid culture rather than on solid medium may enhance bacterial response to microgravity, and also to interactions between microgravity and radiation. It is possible that a longer-term experiment with a higher radiation dose might lead to increased mutation rates. Onboard implementation of a defined radiation dose would be desirable in comparison with unexposed flight control samples. Orbiting for longer periods or at a higher altitude, and interplanetary travel may give higher doses of damaging radiation in addition to extended microgravity exposure when use of a radiation source is not available.

Since *Streptomyces* spp. are notably prone to genetic changes in response to environmental stress [Dyson & Schrempf, 1987], the results show that there is limited potential for mutation when cultures and cells are subjected to spacecraft flight conditions for short periods in low earth orbit.

**ACKNOWLEDGEMENTS**

The collaboration, cooperation and assistance of personnel from GosNIIGenetika (in particular L. Emelianova, L. Novikova and B. Tyagolov) and the Institute for Biomedical Problems (especially E. Ilyin) in Moscow, Russia, were critical to this project and are gratefully acknowledged. In addition we thank staff at NASA Ames Research Center, particularly M. Skidmore, K. Souza, R. Boyle, E. Almeida, V. Vizir, and G. Tverskaya, and others at NASA Headquarters, for their help and support. KL and AS were supported in part by Grant Number P20 RR-16455-06 from the National Center for Research Resources (NCRR), of the National Institutes of Health (NIH) through the Montana State University Undergraduate Scholars Program. KL also received a ‘Life in Space for Life on Earth’ conference student travel grant, and BP was awarded a mentor travel grant by the American Society for Gravitational and Space Biology. The project was funded by NASA grant NCC2-1143.

**REFERENCES**


