PROPOSAL FOR FOTON-M3 EXPERIMENT PLASMID

Spaceflight Effects on Structural Stability and Genetic Information Transfer in Plasmid plJ702 of Actinomycetes Streptomyces lividans 66

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BACKGROUND

Our research, as proposed originally for experiment PLASMID on Foton-M2, was to focus on molecular analysis of *Streptomyces lividans 66* plasmid p1 J702 retention, stability and amplification in response to spaceflight conditions, to supplement and complement the analyses to be completed by the Russian investigators.

1) Using primers for the plasmid marker genes, polymerase chain reaction (PCR) would be performed to confirm that the plasmid marker is present in the strain that bore the plasmid.

2) To determine the plasmid copy number per cell, which may be affected by microgravity/spaceflight conditions, plasmid DNA would be separated from chromosomal DNA using standard molecular cloning techniques. The amount of plasmid DNA would then be quantified, and presence of the plasmid marker would be confirmed by PCR.

3) Denaturing Gradient Gel Electrophoresis (DGGE) could be performed in our laboratory to provide detect point mutations on the plasmid. If chromosomal markers are available, these could also be analyzed for point mutations using DGGE. DNA bands from the DGGE gels would be sequenced using BigDye version 3.1 (ABI) and analyzed using the Sequencer Program. These assays would complement the Restriction Fragment Length Polymorphism (RFLP) analysis (restriction analysis) proposed by the Foton investigators.

4) If suitably preserved and stowed samples can be provided, proteomic analyses would be possible. In addition, if RNA preservation is possible, we could do Real Time PCR (RT-PCR) to assess relative translation of the plasmid under flight vs ground conditions.

To summarize, PLASMID experiment objectives were: 1, to study spaceflight effects on genetic structures of the microorganism; 2, to identify the pattern and mechanism of genetic changes; and, 3, to determine relationships between the changes and specific spaceflight factors, i.e., space radiation and microgravity. The primary goal of this research is to compare the stability and expression of plasmid and chromosomal genes in spaceflight and Ground samples.

Streptomycetes are Gram-positive actinobacteria that grow as a mycelium that differentiates into spore chains. They are responsible for the "earthy" odor of soil, and many produce antibiotics like streptomycin. The plasmid plJ702 was constructed from plasmid pljl 01 _by insertion of the tyrosinase gene from *S. antibioticus* as a phenotypic marker and the thiostrepton resistance gene from *S. azureus* as a selective marker. Thiostrepton is a highly modified multicyclic peptide antibiotic best known as an inhibitor of protein synthesis; it is also a potent activator of gene expression in *S. lividans.*

Our proposed research would significantly enhance the scientific data produced by the Russian experiment. Using published sequence data, nucleic acid primers and all other reagents are readily available. All the equipment required to perform this research is available within the Department of Microbiology at Montana State University, Bozeman.

Specify linkage to the NASA Mission

The Foton M-3 mission provides an opportunity to repeat and enhance the experiment we flew on Foton M-2 and expand our knowledge of how living systems respond to the unique environment of space, i.e. microgravity and space radiation. This experiment will provide information for both chromosomal and plasmid gene expression as well as genetic stability. *Streptomyces* spp. may serve as a sensitive indicator of the effects of spaceflight on microorganisms due to an inherent genetic instability: the effects of long-duration spaceflight missions may be inferred from the fundamental knowledge obtained during relatively short-term spaceflight exposure of *Streptomyces* specimens during the Foton M-3 flight. Detection of genetic changes in *Streptomyces* exposed to spaceflight factors, e.g. radiation and microgravity. would suggest that similar effects may occur in other microorganisms, especially with prolonged exposure to the same factors in long-duration spaceflight. Genetic alterations (mutations) may result in enhanced virulence of pathogenic bacteria. They may also lead to changes in treatment approaches for infections, e.g. the microbes may become more or less susceptible to some antibiotics Changes in the thiostrepton marker would suggest this possibility in other organisms.

FOTON-M2 EXPERIMENT AND FINDINGS

Sixteen Petri dishes (plates) containing bacteria. *Streptomyces lividans 66,* were flown on Foton-M2. Within 30 hours after landing and capsule recovery, samples were received in the laboratory at GosNIIgenetika in Moscow. The cultures failed to progress through their full life cycle;_mycelia did not differentiate and form spores due to lower than nominal temperatures within the Foton capsule and Plasmid experiment container. Laboratory control cultures incubated at 25°C for the duration of the flight matured and differentiated into spores. Since the cultures had not differentiated during incubation at Flight and Ground conditions, the Russian and US investigators agreed that one of each replicate plate (8 out of the 16 that were flown) should be re-incubated in the Moscow laboratory at 28°C until sporulation had occurred. While this re-incubation for both Flight and Ground Controls was done in 1xg conditions, it can be assumed that spaceflight conditions would have influenced the bacteria in the early stages of growth. Samples were cut from agar plates and prepared for shipment to the US within 18 days of return from orbit (R). The cultures were inadvertently shipped on dry ice (instead of wet ice packs) and shipping delays extended the transit time from handover at the Russian lab to arrival at the US lab to about 7.5 days. On arrival, there was still dry ice in the package, and the frozen samples were transferred to a -20°C freezer on the advice of Dr. Tabakov.

In spite of these difficulties, we have been able to subculture many of the clones shipped from Moscow and perform genetic analyses. Spores and/or mycelium were harvested and frozen for later analysis. Population and clonal genetic variability in relation to the plasmid marker genes were investigated under selective and non-selective growth_conditions. Clones derived from flight samples and asynchronous controls showed a similar high frequency loss of the melanin and thiostrepton resistance phenotypes compared to laboratory controls. Initially it was thought that this most likely resulted from_plasmid loss due to sub-optimal temperatures (15-20°C) of the flight and asynchronous controls compared to lab controls (26-28°C). In isolates that retained the plasmid, crude DNA restriction fragment polymorphism (RFLP) analysis (in the Moscow lab) did not reveal any changes in.plJ702 DNA isolated from flight vs. ground asynchronous control clones.

We found that high proportions (around 80%) of clones from flight and asynchronous control samples did not express either marker phenotype (tsr-, mel-), compared to about 30% in lab control clones. When some mel- clones (from flight and asynchronous controls) grown without thiostrepton were incubated with thiostrepton, they produced melanin (mel+).

We have isolated plasmid DNA out of clones from the flight/high inoculum/ISP-thiostrepton plate (Moscow Sample ID 12) that failed to express either of the markers (Fig. 1). PCR amplification and agarose gel electrophoresis has demonstrated the presence of essentially

complete tsr gene sequences and variable sequences of parts of mel genes. Insertions, deletions, and disruption of primer binding sites have been detected after PCR amplification of these gene fragments. Denaturing Gradient Gel Electrophoresis (DGGE) with and without GC clamps is being performed on these amplicons to detect point mutations. Using mutagenic PCR, we have generated clones with single point mutations and demonstrated that DGGE can detect mutations. Failure to amplify the 544bp fragment of the tsr gene (Fig. 1, lane 2) may indicate loss of the plasmid or mutation within one of the primer sequence sites.

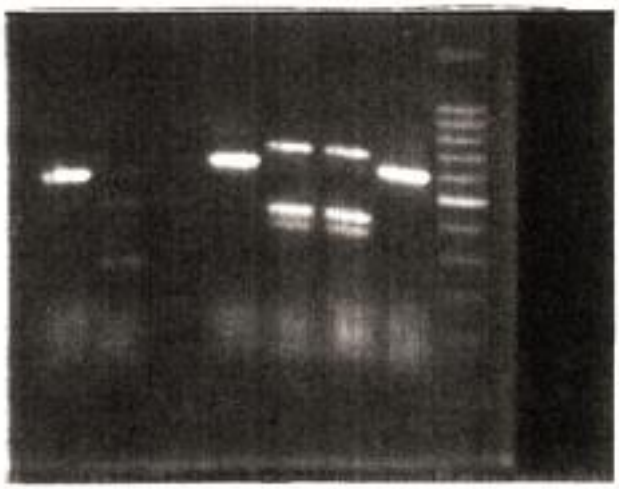

Figure 1: Agarose gel electrophoresis of PCR amplification products from tsr-/mel- clones isolated from Sample 12 (Heavy Inoculum on ISP agar without thiostrepton, flight incubation).

Lane 1: Clone v5, primers tsr2F/tsr4R, product size as expected (544bp).
Lane 2: Clone v2, primers tsr2F/tsr4R no product detected
Lane 3: No sample
Lane 4: Clone v3, primers mel2F/mel5R, single product size as expected (591 bp).
Lane 5: Clone v4, primers mel2F/5R, three product bands suggest genetic changes.
Lane 6: Clone v5, primers mel2F/4R. three product bands suggest genetic changes.
Lane 7: Clone v6, primers mel2F/4R, single product size as expected (591 bp).
Lane 8: 100bp standard ladder

Some clones from Sample 12 grown on medium without thiostrepton fail to produce melanin but when grown on medium containing thiostrepton, melanin is produced. This suggests that there may be a defect in the promoter region of the mel genes that is circumvented in the presence of thiostrepton which is known to be a powerful activator of gene expression.

We are now trying to amplify a section of the tsr gene with a GC clamp to allow DGGE comparison of amplicons from asynchronous control and wild type (original) clones on a single base pair level. We are also using our primer sets to examine different parts of the marker genes. This will enable us to determine that the plasmid has not been incorporated into the chromosome, that concatemers (repetitions of the same sequences) have not been formed, or that no other rearrangements have occurred. We will also attempt plasmid isolation from tsr-, mel- clones from asynchronous controls and a lab control to determine if there are similar changes to those found for sample 12. As a negative control, we will attempt to isolate plasmids from a plasmid-negative control strain provided from GosNIIGenetika to confirm primer/PCR reliability. Plasmid isolation from marker-negative clones from other samples will be attempted to determine if they also contain defective marker plasmid DNA.

Thus, we suspect that specific spaceflight factors such as microgravity and radiation encountered during the Foton-M2 mission may affect mutation frequency of the plasmid marker genes. While factors not specific to spaceflight (e.g. temperature) may have a significant effect on the genetic stability and physiology of the bacteria, our results suggest that this did not result in loss of the plasmid but rather in alteration of the marker genes so that they became defective in expressing the markers. It is most likely that this is the result of point mutations or other genetic

changes such as rearrangements within the plasmid DNA.

PROPOSED RESEARCH FOR FOTON-M3

Aberrations in the Foton-M2 experiment included:

1) pre-flight difficulties in the GosNIIGenetika lab, i.e. a power outage that resulted in the need to use different cultures for flight and ground samples; 2) sub-optimal incubation temperatures on-orbit; 3) shipment of samples at freezing rather than refrigeration temperatures; and 4) extended shipping times from Moscow to Bozeman. We therefore propose that the experiment should be repeated essentially as it was intended to be done for Foton-M2, taking care to avoid similar aberrations.

We will use the techniques and materials developed for the Foton-M2 experiment to achieve our original objectives. It is anticipated that with directly comparable inocula being used for flight and ground control samples, and optimal on-board temperatures, the bacteria will complete their growth cycle which will maximize the effects of radiation and other spaceflight factors such as microgravity. Plasmid DNA mutations resulting in the loss of melanin and/or thiostrepton resistant phenotypes will be detected by denaturing gradient gel electrophoresis (DGGE). Chromosomal changes, chloramphenicol resistance or impaired DNA repair mechanism, could also be analyzed by DGGE if a change in phenotype is detected. We will also examine the effect of thiostrepton on expression of the mel+ phenotype.

We will plan to ship whole agar plates rather than agar plugs as was originally anticipated for the Foton-M2 experiment. In addition, we will take steps to ensure that the samples are transported correctly at refrigeration temperatures (with wet ice) and that shipping delays are avoided so that they can reach our lab within 2-3 days after they are handed over from GosNIIGenetika. Our experience with the Foton-M2 experiment will allow us to proceed efficiently with sample processing and obtain reliable results in a timely fashion.