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The effect of space flight on the production of actinomycin D by *Streptomyces plicatus*

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The effect of space flight on production of the antibiotic actinomycin D by *Streptomyces plicatus* WC56452 was examined onboard the US Space Shuttle mission STS-80. Paired space flight and ground control samples were similarly prepared using identical hardware, media, and inoculum. The cultures were grown in defined and complex media under dark, anaerobic, thermally controlled (20°C) conditions with samples fixed after 7 and 12 days in orbit, and viable residuals maintained through landing at 17 days, 15 h. Postflight analyses indicated that space flight had reduced the colony-forming unit (CFU) per milliliter count of *S. plicatus* and increased the specific productivity (pg CFU^{-1}) of actinomycin D. The antibiotic compound itself was not affected, but its production time course was altered in space. Viable flight samples also maintained their sporulation ability when plated on agar medium postflight, while the residual ground controls did not sporulate.
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Introduction

The weightless environment experienced during space flight has been repeatedly shown to stimulate *in vitro* microbial growth [2,7,9,10,13–15]. Based on previously reported observations to this effect, it was hypothesized that secondary metabolite production might be affected as a consequence. Our initial investigation showed the specific productivity of the antibiotic monorden by *Humicola fuscoatra* in solid-state fermentation on two types of media (defined and complex) to be increased by up to 190% in space [11]. Gravity-dependent extracellular mass transport phenomena are thought to be responsible for indirectly affecting microbial physiology by allowing altered chemical gradients to develop in the immediate microenvironment of a cell under weightless conditions. In the absence of buoyancy and sedimentation, mass transport is reduced to diffusion only, which is theorized to reduce net nutrient availability to and byproduct dispersion rates from the cells [9]. In contrast to the fluid dynamics associated with the highly viscous agar-based system used to produce monorden [11], the experimental objectives here were to obtain similar baseline data on antibiotic production in a liquid suspension culture. Two types of media were again used to collect additional data for correlating the results with differing nutrient and byproduct molecular diffusion coefficients.

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Materials and methods

Microorganism

Streptomyces plicatus WC56452 used to produce actinomycin D was obtained from the Bristol-Myers Squibb Culture Collection (Wallingford, CT).

Culture media

S. plicatus WC56452 was maintained in 2 ml of seed medium before being inoculated on orbit into one of the two media, designated F1A (complex) and DF1 (defined). The seed medium contained (g l^{-1}): starch, 20; D-glucose, 5; N-Z case (Quest International, Norwich, NY), 3; yeast extract (Difco, Sparks, MD), 2; fish meat extract (Mikuni Kagaku-Sangi, Tokyo, Japan), 5; and $CaCO_3$, 3. F1A contained (g l^{-1}): starch, 20; D-glucose, 10; Pharmamedia (Traders Protein, Fort Worth, TX), 10; yeast extract (Difco), 3; N-Z amine A (Quest International), 3; and $CaCO_3$, 3. DF1 contained (g l^{-1}): D-fructose, 40; L-glutamic acid·HCl, 4.5; L-histidine·HCl·H_2O, 1; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.02; and $FeSO_4 \cdot 7H_2O$, 0.02. Ethanol (100%) was used as the fixative agent.

Space flight protocol

The experiments were carried out in a device called the Bioprocessing Module (BPM) designed to contain isolated fluids for launch and subsequently mix them in the absence of gravity. The BPM consists of four syringes attached to a manually operated valve that can be configured for interconnection to allow activation and sampling (Figure 1). The BPM is sealed inside two layers of FEP Teflon™ bags for additional levels of containment. No gas exchange or agitation was provided. In-flight inoculation and two

Figure 1 BPM photograph. Sterile medium (6 ml) was loaded into syringe A, inoculum in seed medium (2 ml) in B, and a fixative (ethanol, 1.5 ml each) in C and D.

sampling time points were achieved as follows: at ~52 h before launch, syringe A was loaded with 6 ml of sterile medium, B with 2 ml of inoculum in seed medium, C and D with 1.5 ml ethanol, and placed at 20°C. Two BPMs contained the complex (F1A) medium and two contained the defined (DF1) medium. Matched ground controls were also loaded and treated identically. The space shuttle Columbia lifted off at 2:55 p.m. EST on November 19, 1996. The four flight and four ground BPMs were manually activated ~56 h after launch by transferring the contents of syringe A into B and back into A (to mix), then distributed, half (4 ml each) into A and B. The cultures continued to be maintained at 20°C under anaerobic conditions. At ~7 days, 3 h, the first sample time point was fixed by transferring 1.5 ml from syringe B into C, and at ~12 days, 6 h, 1.5 ml was fixed from A into D. All BPMs were then placed at 6°C for the duration of the mission. The residual 2.5-ml samples in syringes A and B remained viable for return. Landing occurred at 6:49 a.m. EST on December 5 at the Kennedy Space Center in Florida, and STS-80 became the longest shuttle mission to date (17 days, 15 h, 53 min). All flight and ground samples were placed on dry ice at 10:50 a.m. EST and shipped overnight to Bristol-Myers Squibb for analysis.

Postflight extraction and analytical methods

The 1.5-ml fixed ground and space samples were extracted using 1.5 ml of ethyl acetate for 1 h. The extracts were centrifuged at 3000 rpm for 15 min and evaporated to dryness under a stream of nitrogen. Extracts from duplicate samples were combined in order to ensure that adequate amounts of actinomycin D were available for the HPLC photodiode array study. Viability counts (CFU ml^{-1}) were determined from the residual, nonfixed samples remaining in the BPM syringes A and B. Aliquots of 0.1 ml from syringes A and B were plated onto asparagine–glycerol agar to compare the postflight sporulation ability of the *S. plicatus* space flight and ground control samples.

The quantity of actinomycin D present in each sampling time point was determined by HPLC analysis using a Rainin Microsorb Short-one C-18 column (3.9×100 mm) and the solvent system of Meta O as described by Hook *et al* [4]. The flow rate was 1.2 ml min^{-1} and the detector wavelength was 254 nm. The extract was dissolved in 100 μl of DMSO and 50 μl was injected into HPLC. Antibiotic amounts were quantified by comparing peak areas using a standard actinomycin D plot.

Chromatography for LC-MS analysis was performed using a Hewlett-Packard Model 1090 HPLC equipped with a diode array detector. A Rainin Microsorb Short-one C-18 column was used with a mobile phase of CH_3OH:CH_3CN:20 mM ammonium acetate (1:1:1) with a flow rate of 1 ml min^{-1}. Mass spectra were obtained using a Hewlett-Packard Model 5989A mass spectrometer equipped with a thermospray interface to the HPLC.

Results and discussion

The STS-80 space flight and ground control experiments were carried out as planned with no anomalies. The BPM hardware supported bacterial growth and antibiotic production, albeit under less than optimal fermentation conditions without agitation or aeration. The simple design parameters of the BPM facilitated manual operation in space and allowed researchers to isolate gravity as an independent variable, but absolute productivity levels under these conditions are not directly comparable to typical terrestrial processing techniques.

S. plicatus CFU per milliliter and sporulation

As shown in Table 1, viable cell counts in the complex medium of the day-17 sample exceeded those in defined medium. The concentration of viable cells in the ground controls, however, was about threefold higher than the space samples in both media. The residual viable space samples sporulated profusely after plating

Table 1 Viable cell count, volumetric productivity, and specific productivity of actinomycin D of the space and ground samples on day 17

Sample	Viable cell count (CFU ml^{-1})	Volumetric productivity ($\mu g\ ml^{-1}$)	Specific productivity (pg CFU^{-1})
Defined medium, space shuttle	4000	0.34	85.5
Defined medium, ground control	12,100	0.26	21.6
Complex medium, space shuttle	59,000	0.39	6.7
Complex medium, ground control	174,000	0.173	0.99

them postflight on asparagine–glycerol agar and incubating the cultures at 28°C for 3 days while the ground controls lost their ability to sporulate when similarly plated. Previous studies showed *Bacillus subtilis* spore formation to also be altered in-flight, increasing in one case but decreasing in another [14,15]. Rationale for the observed changes in sporulation has yet to be established, but may be linked to gene regulation associated with differentiation and/or secondary metabolite production processes [1,5,12,18]. One explanation may be related to the influence of growth rate on the sporulation of *Penicillium chrysogenum* observed in terrestrial fermentation [17]. Slower growth rate during fermentation has a positive effect on sporulation of *P. chrysogenum*. Since growth of the space flight samples was slower than that of the ground controls, it was not surprising to observe that the space flight samples had better sporulating ability than the ground samples.

Actinomycin D production

Production of actinomycin D in the flight samples and ground controls was confirmed by LC-MS analysis. The maximum specific productivity of the space and ground samples in terms of amount of antibiotic produced per weight of extract was very similar at 0.93 and 0.96 $\mu g\ mg^{-1}$, respectively, in the defined medium (Figure 2A). In the complex medium (Figure 2B), however, the maximum space sample (1.02 $\mu g\ mg^{-1}$ extract) was 115% higher than the ground sample (0.47 $\mu g\ mg^{-1}$ extract).

When comparing specific productivity of the day-17 cultures in terms of the amount of antibiotic produced per viable cell, the space samples were considerably higher than the matched ground controls in both media, with a 296% increase in the defined medium and a 577% increase in the complex (Table 1). These increases in productivity may also be related to growth rate. It has been well documented in terrestrial fermentation that limitations in culture growth often lead to increased secondary metabolite productivity [3,6,16,19,20]. It appears that a certain low specific growth rate must be maintained to allow an organism to exploit its full capacity for antibiotic biosynthesis. Slower growth of the space flight cultures may have resulted in higher specific productivity yields than faster-growing ground control cultures. A reduction in extracellular mass transport due to the weightless conditions experienced in space may be responsible for the decreased growth rate, as all other primary potential causal factors (medium, temperature, time, containment) were kept constant.

Production time course

In both the defined (DF1) and complex (F1A) media, the volumetric and specific productivity of actinomycin D in the ground samples reached a maximum on day 7, the first sampling day, and productivity dropped with further incubation (Figure 2A and B). However, the volumetric and specific productivity of the space sample grown in the defined medium continued to increase with culture age throughout the 17-day mission (Figure 2A). In complex medium, the volumetric and specific productivity of the space samples reached a maximum on day 12 and, after stowage at 6°C, indicated a slight drop in productivity on day 17 (Figure 2B). These rates suggest that the production time course may have not yet peaked in either of the flight sample groups, indicating that additional duration in space is needed to fully evaluate the process kinetics.

Of particular interest in these findings were the variations noted in the time course of antibiotic production in space and the unique responses observed to the two different types of growth media.

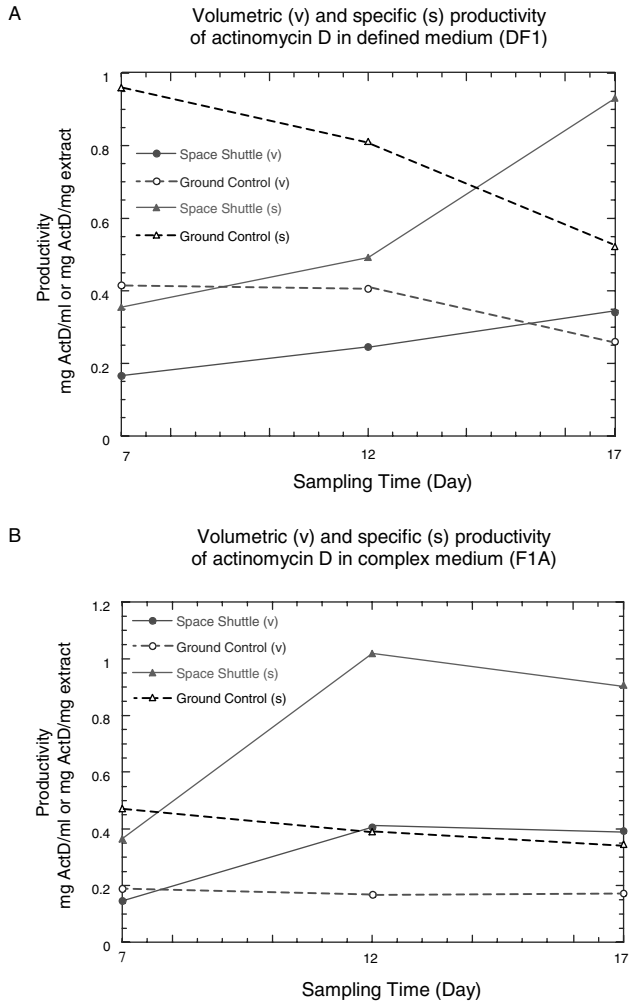

Figure 2 Time course for volumetric (v, $\mu g\ ml^{-1}$) and specific (s, $\mu g\ mg^{-1}$ extract) productivity of actinomycin D by *S. plicatus* WC56452 in defined (A) and complex (B) medium. (●) Space shuttle, volumetric productivity; (○) ground control, volumetric productivity; (▲) space shuttle, specific productivity; (△) ground control sample, specific productivity.

Medium-dependent microbial responses to space flight have also been noted by Mennigmann and Heise [14] and Lam *et al* [11], further suggesting that the underlying mechanisms acting on biological systems may be indirectly related to transport coefficients of the differing molecular constituents in the nutrients and byproducts.

Future research

In addition to the primary sample analyses, parallel efforts are ongoing towards optimizing a fermentor for use in a weightless environment and evaluating potential commercial applications of this research. Computational techniques are also being developed in an attempt to characterize the role that gravity plays on cellular level processes [8]. A follow-on experiment was carried out onboard STS-95 in October 1998 to evaluate a new, partially aerobic, batch fermentor design. Ground based tests showed that actinomycin D production in this device was increased by ~20-fold over the BPM. The flight results from this experiment will be reported in conjunction with research utilizing a fed batch reactor system that is currently aboard the International Space Station (ISS 8A Increment). The latest reactor design resulted in an additional ~10-fold improvement over the batch processor in ground based productivity, or ~200-fold over the original BPM levels.

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