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Secondary metabolism in simulated microgravity: β-lactam production by *Streptomyces clavuligerus*

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Rotating bioreactors designed at NASA's Johnson Space Center were used to simulate a microgravity environment in which to study secondary metabolism. The system examined was β-lactam antibiotic production by *Streptomyces clavuligerus*. Both growth and β-lactam production occurred in simulated microgravity. Stimulatory effects of phosphate and L-lysine, previously detected in normal gravity, also occurred in simulated microgravity. The degree of β-lactam antibiotic production was markedly inhibited by simulated microgravity.

Keywords: simulated microgravity; gravity; β-lactam antibiotics; *Streptomyces clavuligerus*; space microbiology

Introduction

The adaptability of microorganisms to environmental influences poses two important questions concerning space flight: (i) how do microorganisms affect crew and spacecraft during extended flights? and (ii) how does spaceflight affect microorganisms? We have begun studies to address the second question. Studies in space have been done on the effects of microgravity on microbial growth, resistance to radiation, phage induction [6,8], susceptibility to antibiotics, rate of conjugation [1,7,9,12], susceptibility to vacuum and UV irradiation [7,11], phage productivity and survival rate [14], and cell morphology [11]. Although the results are not totally in agreement, microgravity seems to increase growth of bacteria and their resistance to certain antibiotics [8]. No reports have been published on whether microgravity affects secondary metabolism of microorganisms. In the present work, we examined, as a model system, the biosynthesis of β-lactam antibiotics and its regulation in the filamentous bacterium *Streptomyces clavuligerus*. A simulated microgravity (SMG) environment was provided by the use of rotating bioreactors designed at the National Aeroneutics and Space Administration's (NASA's) Johnson Space Center. We were interested in three questions: (i) Does β-lactam antibiotic production occur in SMG? (ii) Do stimulatory effects of effector compounds, previously noted in normal gravity, occur in SMG? (iii) Does SMG have an effect on the level of β-lactam antibiotic production? Experiments on these questions are the subject of this communication.

Materials and methods

Microorganisms

S. clavuligerus NRRL 3585 (ATCC 27064) is a Gram-positive filamentous soil bacterium. This actinomycete produces a variety of β-lactam antibiotics, namely penicillins, cephalosporins (including cephamycin C), and clavulanic acid; the main product is cephamycin C. Spores were produced by culturing *S. clavuligerus* on an agar medium containing (g L^{-1}): yeast extract, 4; malt extract, 10; glucose, 4; Bacto-agar (Difco Laboratories, Detroit, MI, USA), 20; pH was adjusted to 7.3 with KOH. Spores were harvested at 7 days and stored at −80°C in 20% (v/v) glycerol. *Escherichia coli* ESS, a mutant of *E. coli* B that is supersensitive to β-lactams [13], was the assay organism for β-lactam antibiotics.

Fermentations

The seed medium was tryptic soy broth (TSB, Gibco Laboratories, Madison, WI, USA), 30 g L^{-1}. The basic chemically defined fermentation medium contained (g L^{-1}): glycerol, 10; L-asparagine, 2.0; L-lysine, 1.83; 3-(N-morpholino)propanesulfonic acid (MOPS), 21; $MgSO_4 \cdot 7H_2O$, 0.6; K_2HPO_4, 3.5 and trace salts solution (containing $FeSO_4 \cdot 7H_2O$, 1.0; $MnCl_2 \cdot 4H_2O$, 1.0; $ZnSO_4.H_2O$, 1.0 and $CaCl_2$, 1.0), 1 ml L^{-1}. The pH was adjusted to 6.8 with KOH before autoclaving the medium. In certain experiments on nutrient regulation, L-lysine was increased ten-fold to 18.3 g L^{-1} (=100 mM) or K_2HPO_4 was increased six-fold to 21 g (120 mM).

Bioreactors

The bioreactors (Figures 1, 2) were originally designed at NASA's Johnson Space Center to create a low-turbulence, low-shear environment that would allow human cells to grow and assemble into three-dimensional constructs. Like other bioreactor systems, the NASA vessels are rotated such that the cell sedimentation associated with gravitational forces is balanced by the centrifugal forces caused by the rotation. However, in conventional bioreactor systems, the turbulence associated with movement of the fluid environment induces shear stress, a potentially damaging force exerted on cells when they encounter impeller blades, reactor walls, fluid media and air bubbles. The bioreactors used here allow nutrients to be circulated and waste products to be removed, but at a shear stress less than one-

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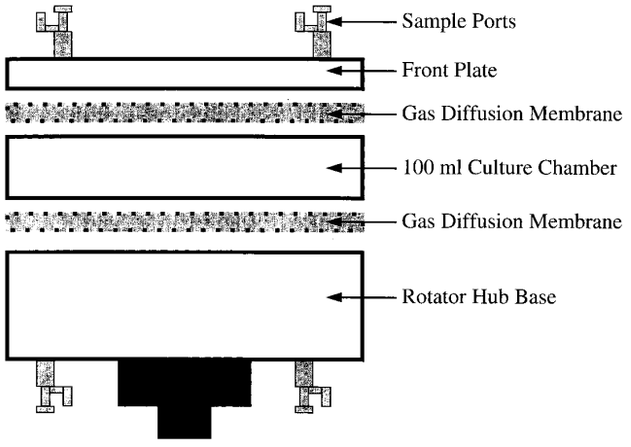

Figure 1 The dual high aspect rotation vessel (dHARV).

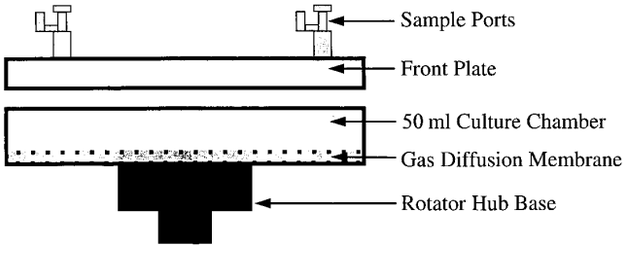

Figure 2 The single high aspect rotating vessel (sHARV).

tenth of those typical of conventional bioreactors which are 3–10 dynes cm^{-2}.

We used two types of 'high-aspect' rotating vessels (HARVs). For both types, the vessel cylinder was filled completely with medium and cells and rotated at 60 rpm about either a horizontal or a vertical axis (see below). The residual shear experienced by cells in these conditions is typically 0.2 dynes cm^{-2} [10]. The prototype dual-HARV (dHARV) (Figure 1) consists of a 100-ml cell growth chamber sandwiched between two membranes. The outer membrane is permeable to gas and exposed to the environment. The rear membrane is permeable to liquid and gas, and is exposed to a 150-ml growth medium reservoir located behind the growth chamber. The prototype vessel was modified to increase airflow by drilling a hole in the back of the medium reservoir, thus allowing air from the air pump to flow through the rotator hub to a gas permeable membrane located at the rear of the growth chamber. The second vessel type, the single-HARV (sHARV) (Figure 2), has a 50-ml growth chamber and has only one gas-permeable membrane at the rear of the vessel facing the rotator hub. The sHARV can be positioned horizontally so that the axis of rotation is perpendicular to the gravity vector or vertically with the axis being parallel to the gravity vector. The horizontal position provides an environment simulating microgravity. On the other hand, the vertical position provides a near normal gravity environment (actually slightly higher than normal gravity due to the centrifugal force of the rotating motion).

Incubation and sampling conditions

Seed medium (50 ml) in 500-ml Erlenmeyer flasks was inoculated with 1.0 ml of spore suspension, prepared as described above, and incubated at 30°C on a rotary shaker (5.1-cm diameter) at 220 rpm for 38–40 h.

The HARVs were autoclaved and filled with autoclaved chemically-defined medium by syringe (100 ml for the dHARV and 50 ml for the sHARV). The HARVs then were inoculated by syringe with 5 ml (dHARV) or 2.5 ml (sHARV) of seed culture and corresponding volumes of medium were removed from the reactor. The HARVs were rotated at 60 rpm at 30°C in an incubator room that also housed the normal gravity controls. Since we had only one dHARV available at the start of this work, the vessels used as normal gravity controls were 500-ml Erlenmeyer flasks, containing various volumes of media (see Results), inoculated with 5% (v/v) of seed culture, and incubated at 30°C on a rotary shaker (5.1 cm diameter) at 120 rpm for up to 10 days. Later in our work, when duplicate sHARVs became available, flask controls were replaced by normal gravity controls conducted in the second sHARV. Conducting SMG and normal gravity controls in the same type of vessel gave us much better control of experiments.

Samples were collected from the HARVs (and flasks) every 2 days. They were drawn by syringe (3 ml from the dHARV, 1 ml from the sHARV) and 10.5 ml from the flasks) and corresponding volumes of fresh medium injected immediately thereafter.

Assays

Growth in the fermentation systems was determined by absorbance with a Klett Summerson colorimeter (Klett Manufacturing Co, New York, NY, USA) using a red filter. For these measurements, 1.0 ml of whole broth was added to an equal volume of 2.5 N HCl. Water was added to 10 ml and the suspension treated in the Branson Sonifier cell disruptor model 200 (Branson Ultrasonics Co, Danbury, CT, USA) for 30 s. The absorption of the sonicated suspension was determined in the range of 50–150 Klett units. If the suspension was too dense, it was diluted with water and the observed Klett units were multiplied by the dilution factor. Under these conditions, Klett units are linearly related to dry cell weight (DCW) and a DCW of 1 g L^{-1} is equivalent to 270 Klett units.

The production of β-lactam antibiotics was determined with the agar plate-disk diffusion assay with the assay strain seeded in Luria-Bertani (LB) agar; the agar concentration was 8 g L^{-1}. Cephalosporin C was used as β-lactam antibiotic standard and *E. coli* ESS as the assay organism. Repeated assays showed a maximum variation of 12%.

Results

β-Lactam production in simulated microgravity

When these studies began, only one modified HARV was available to us. Thus, we used a normal gravity flask control system as a measure of reference. We found that *S. clavuligerus* could grow in the modified dHARV, but at a much lower rate (5–10%) than in flasks under the high aeration condition optimal for production of β-lactam antibiotics. Since the dHARV apparently was operating under

a growth-limiting oxygen supply, we increased the volume of medium in the flasks to slow growth [2], through oxygen limitation to a rate approximating that occurring in the modified dHARV. Growth and antibiotic production in the modified dHARV were roughly equivalent to that in a 500-ml flask containing between 300 and 400 ml of medium, rotating on a shaker with a 5.1 cm orbit at 120 rpm. A further experiment, in which performance in the dHARV was compared to 500-ml flasks containing 350 ml medium, showed approximately similar growth and β-lactam production in these two types of vessel.

Effects of nutrients on cephalosporin production in SMG

When oxygen is limited in flask cultures, a high concentration of phosphate (120 mM) has been shown to stimulate β-lactam formation by *S. clavuligerus* [2]. In the present work, high phosphate stimulated secondary metabolism in the modified dHARV under SMG.

High concentrations of L-lysine (eg 100 mM) are known to stimulate β-lactam production by *S. clavuligerus* in flasks [3]. Lysine is stimulatory because it is converted to the β-lactam precursor, L-α-aminoadipic acid [4,5]. Furthermore, we have recently found that lysine induces lysine ε-aminotransferase (Rius and Demain, unpublished), the first enzyme of cephamycin biosynthesis in *S. clavuligerus*. In the present work, we found 100 mM L-lysine to be stimulatory also under SMG in the modified dHARV.

Effect of SMG on level of β-lactam production

A critical determination of the effect of SMG on the quantitative aspect of antibiotic production by *S. clavuligerus* had to await the availability of two identical sHARVs. One of these was used in the usual horizontal orientation to simulate microgravity and the other in the vertical orientation to provide a normal gravity control. We found that SMG inhibits β-lactam production (Figure 3). Although growth extent was somewhat less in SMG than in normal gravity (0.40 *vs* 0.67 g L^{-1} DCW, respectively), the maximum specific β-lactam production was several times higher in normal gravity than in SMG, ie 4.2 *vs* 1.7 (μg antibiotic mg^{-1} DCW) respectively.

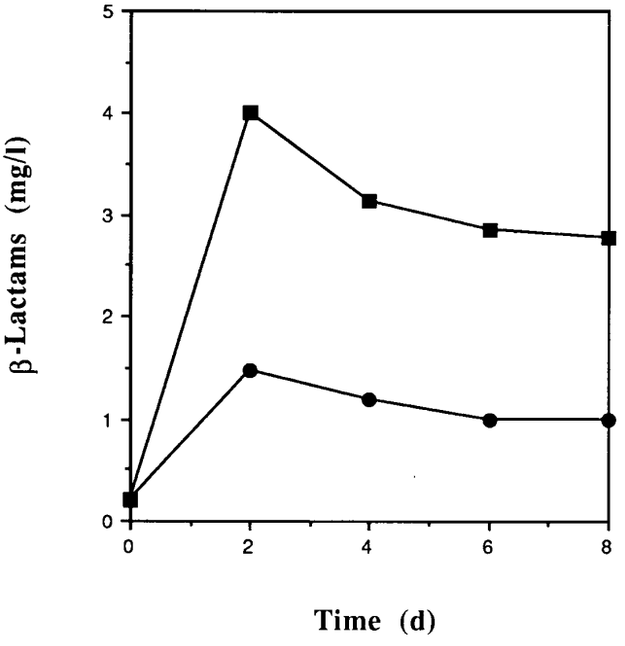

Figure 3 Effect of SMG on β-lactam production in the sHARV. ■, normal gravity; ●, SMG.

Discussion

The present work clearly answered the questions posed in the Introduction: (i) *S. clavuligerus* can produce β-lactam antibiotics in SMG; (ii) the stimulatory effects of additional phosphate and high lysine concentrations, previously observed under normal gravity in flask cultures, are also evident in SMG; (iii) the use of two identical sHARVs, which became available during this work, gave us better control of the experiments and revealed that SMG exerts a profound negative influence on β-lactam production by *S. clavuligerus*. In future work, we shall determine by HPLC whether the ratio of cephalosporins produced in SMG is the same as in normal gravity.

This example of secondary metabolism was chosen as a model system to help us to predict the effects of space flight upon secondary metabolite biosynthesis and its regulation. Such metabolism could have significance for the health of space travellers on long term flights, since many secondary metabolites are highly toxic or carcinogenic and some are volatile, further increasing exposure of the crew. The inhibition of secondary metabolism in *S. clavuligerus* observed here suggests that production of secondary metabolites may not be a problem in space. However, many more examples of secondary metabolic systems will have to be examined before we can conclude that there is no special danger concerning secondary metabolism under microgravity conditions. Such efforts are ongoing in these laboratories.

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