A. Fang[1], D. L. Pierson[2], S. K. Mishra[3] and A. L. Demain[1],*

# Growth of Streptomyces hygroscopicus in rotating-wall bioreactor under simulated microgravity inhibits rapamycin production

**Abstract** Growth of Streptomyces hygroscopicus under conditions of simulated microgravity in a rotating-wall bioreactor resulted in a pellet form of growth, lowered dry cell weight, and inhibition of rapamycin production. With the addition of Teflon beads to the bioreactor, growth became much less pelleted, dry cell weight increased but rapamycin production was still markedly inhibited. Growth under simulated microgravity favored extracellular production of rapamycin in contrast to a greater percentage of cell-bound rapamycin observed under normal gravity conditions.

## Introduction

Many physical and chemical factors have been examined for their effect on microorganisms, but gravity has only been studied to a limited extent. Studies in space have been done on the effects of microgravity on microbial growth, resistance to radiation, phage induction (Mattoni et al. 1971; Mishra and Pierson 1992), susceptibility to antibiotics, rate of conjugation (Cliferri et al. 1986; Mennigmann and Lange 1986; Moatti et al. 1986; Tixador et al. 1985), susceptibility to vacuum and UV irradiation (Mennigmann and Lange 1986; Spizizen et al. 1975), phage productivity and survival rate (Zhukov-Verezhnikov et al. 1971), and cell morphology (Spizizen et al. 1975). Although the results from space flights are not totally in agreement, microgravity seems to increase growth of bacteria and their resistance to certain antibiotics (Mishra and Pierson 1992). Not only is

A. Fang · A. L. Demain*
Fermentation Microbiology Laboratory, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
e-mail: demain@mit.edu
Tel.: +1-617-253-1711
Fax: +1-617-253-8699

D. L. Pierson[1] · S. K. Mishra[2]
[1]NASA and [2]Universities Space Research Association, Johnson Space Center, Houston TX 77058, USA

the lag phase shorter and the final density higher but the growth rate may be increased in space (Kacena et al. 1999). Only few reports have been published on whether microgravity affects secondary metabolism of microorganisms (Lam et al. 1998). However, ground-based studies are ongoing in this laboratory to determine whether simulated microgravity (SMG) affects the production of secondary metabolites. An SMG environment is provided by the use of a rotating-wall bioreactor (RWB) designed at the National Aeronautics and Space Administration's (NASA's) Johnson Space Center. We had found that production of gramicidin S by *Bacillus brevis* was unaffected by growth in the RWB under SMG conditions (Fang et al. 1997a). On the other hand, SMG markedly inhibited production of cephamycin C by *Streptomyces clavuligerus* (Fang et al. 1997b) and microcin B17 formation by *Escherichia col i* (Fang et al. 1997c). In the present work, we examined, as a further experimental system, the biosynthesis of rapamycin by the filamentous bacterium *Streptomyces hygroscopicus*. We were interested in the answers to the following three questions: (i) Does rapamycin production occur in SMG? (ii) Does SMG have an effect on rapamycin production? (iii) Does SMG affect the site of rapamycin accumulation?

Rapamycin is a remarkable molecule possessing many activities: immunosuppression (Sehgal et al. 1994), antifungal (Vezina et al. 1975; Wong et al. 1998), antitumor (Eng et al. 1984) and reversal of multidrug resistance (Arceci et al. 1992). It has recently been approved by FDA as an immunosuppressant. It is a 31-membered macrolide made from acetate, propionate, shikimate, L-methionine and L-lysine (via pipecolate) (Paiva et al. 1991; 1993a; 1993b; McAlpine et al. 1991).The genetics and biosynthesis of rapamycin have been reviewed (Reynolds and Demain, 1997).

**Materials and methods**

Microorganism

*Streptomyces hygroscopicus* ATCC 29253 was used to prepare a spore suspension as previously described (Kojima et al. 1995). The spore suspension was stored at -80°C.

Seed cultivation

A seed culture was started by adding 1 ml of a thawed spore suspension into a 250 ml baffled Erlenmeyer flask containing 25 ml of a seed medium consisting of (g/l) soluble starch 10; Bacto-peptone (Difco Laboratories, Detroit, MI) 6; yeast extract (Difco) 6; Casamino acids (Difco) 1.5; $MgSO_4 \cdot 7H_2O$ 0.5; and $K_2HPO_4$ 1.0. The pH was at 7.0 to

7.3. Incubation was at 28 °C for 2 days on a rotary shaker (2 inch diameter, 220 rpm). The resulting seed culture was used at 4% in the fermentation medium.

## Fermentation

The chemically-defined medium of Kojima and Demain (1998) was used for these studies. It contains fructose, mannose, L-aspartate, L-arginine, L-histidine and mineral salts. In addition, L-lysine was added at 10 g/l. The sugars were autoclaved apart from the rest of the medium and pH adjusted to 6.0. Fermentations were conducted for 12 days in RWBs using either the SMG configuration or that for normal gravity (NG). Flasks were also used (baffled and unbaffled 250 ml Erlenmeyers) at 25 ml medium per flask, incubated at 30 °C on a rotary shaker for 12 days.

## Rotating Wall Bioreactor (RWB )

The bioreactors used in these studies 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-tenth of those typical of conventional bioreactors which are 3-10 dynes/cm$^2$. 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$^2$(Prewett et al. 1993). The RWB has a 50 ml growth chamber and a gas-permeable membrane at the rear of the vessel facing the rotator hub. The RWB 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 (SMG). On the other hand, the vertical position provides a near normal gravity (NG) environment (actually slightly higher than normal gravity due to the centrifugal force of the rotating motion).

## Assays

3

Culture broth was centrifuged and extracted by methanol and ethyl acetate as described previously (Kojima and Demain 1998). Dry cell weights and bioassays were done as in our previous paper (Kojima et al. 1995).

## Results

Effect of growth in SMG on rapamycin production

Cells were grown in the RWB under conditions of SMG and NG. Growth was quite different in these two environments in that one or more large pellets formed in SMG but the growth was much more uniform and dispersed in NG. Table 1 shows that the amount of growth and rapamycin production was much lower in SMG than in NG. The specific production of rapamycin decreased by about 90% in SMG.

Effect of growth in SMG in the presence of beads

Because of the pelleting of the cells in SMG, an attempt was made to decrease this morphological effect by adding 25 Teflon beads to the RWB. This modification did decrease pellet formation and increased growth in SMG (Table 2); indeed the difference in growth between SMG and NG was minimal in the presence of beads. However rapamycin production was still markedly inhibited (by about 80%) by growth in SMG.

Effect of growth in SMG on extracellular vs. cellular location of rapamycin

Rapamycin is normally found both in the medium and associated with cells when grown in flasks under NG conditions. As shown in Table 3, about 2/3 is associated with cells and 1/3 is in the medium. We studied whether growth in the RWB would have any effect on this distribution. It was found (Table 3) that there was a shift in SMG in favor of an extra-cellular location; i.e. 66% in the culture supernatant and 34 % in the cell pellet. Under NG in the RWB, the shift was much less significant. When the reactors were run in the presence of beads, the distribution of rapamycin was virtually identical to that in their absence.

## Discussion

The present work clearly answered the questions posed in the Introduction: (i) *S. hygroscopicus* can produce rapamycin in SMG; (ii) production is inhibited by SMG; (iii)

4

the site of rapamycin accumulation is modified to a moderate extent towards an extracellular location. This and the three previous examples of secondary metabolism which we examined were chosen as model systems to help to predict the effects of space flight upon secondary metabolite biosynthesis and its regulation. Such metabolism could have significance for the health of space travelers on long term flights, since many secondary metabolites are highly toxic or carcinogenic, and some are volatile, further increasing exposure to crew. The inhibition of three of the four secondary metabolic processes by SMG suggests that production of secondary metabolites may not be a problem in space. However, more secondary metabolic systems will have to be examined on space flights (Lam et al. 1998) before we can conclude that there is no special danger concerning secondary metabolism under microgravity conditions.

We had earlier observed an almost complete shift in the site of microcin B17 accumulation from cells, as seen in flasks, to the extracellular fluid in the RWB. However, the shift did not appear to be caused by SMG, since it was also observed in the RWB run in NG. We wanted to determine whether this phenomenon was specific for MccB17 or is more general in nature. In the present work, we observed a similar shift towards extracellular location of rapamycin during growth in the RWB, but it was much less dramatic in degree than observed with microcin B17. Such a moderate shift would probably not constitute a health risk for space travelers. Further work is planned on this phenomenon.

## Acknowledgments

# References

Arceci RJ, Stieglitz K, Bierer BE (1992) Immunosuppressants FK506 and rapamycin function as reversal agents of the multidrug resistance phenotype. Blood 80: 1528-1536

Ciferri O, Tiboni O, Di Pasquale G, AM Orlandoni AM, Marchesi ML (1986) Effects of microgravity on genetic recombination in *Escherichia coli*. Naturwissenschaften 73: 418-421.

Eng CP, Sehgal SN, Vezina C (1984) Activity of rapamycin (AY-22,989) against transplanted tumors. J Antibiot 37: 1231-1237

Fang A, Pierson DL, Mishra SK, Koenig DW, Demain AL (1997a) Gramicidin S production by *Bacillus brevis* in simulated microgravity. Curr Microbiol 34: 199-204

Fang A, Pierson DL, Mishra SK, Koenig DW, Demain AL (1997b) Secondary metabolism in simulated microgravity: β–lactam production by *Streptomyces clavuligerus*. J Indust Microbiol Biotechnol 18: 22-25

Fang A, Pierson DL, Mishra SK, Koenig DW, Demain AL (1997c) Effect of simulated microgravity and shear stress on microcin B17 production by *Escherichia coli* and on its excretion into the medium. Appl Environ Microbiol 63: 4090-4092

Kacena MA, Merrell CA, Manfredi B, Smith EE, Klaus DM, Todd P (1999) Bacterial growth in space flight: logistic growth curve parameters for *Escherichia coli* and *Bacillus subtilis*. Appl Microbiol Biotechnol 51: 229-234

Kojima I, Demain AL (1998) Preferential production of rapamycin vs. prolylrapamycin by *Streptomyces hygroscopicus*. J Indust Microbiol 20: 309-316

Kojima I, Cheng YR, Mohan V, Demain AL (1995) Carbon source nutrition of rapamycin biosynthesis in *Streptomyces hygroscopicus*. J Indust Microbiol 14: 436-439

Lam KS, Mamber SW, Pack EJ, Forenza S, Fernandes PB, Klaus DM (1998) The effects of space flight on the production of monorden by *Humicola fuscoatra* WC 5157 in solid state fermentation. Appl Microbiol Biotechnol 49: 579-583

Mattoni RHT, Ebersold WT, Eiserling FA, Keller EC, RomigWR (1971) Induction of lysogenic bacteria in the space environment. In: Saunders JF (ed) The Experiments of Biosatellite II. NASA SP 204, NASA, Washington, DC. pp 309-324

McAlpine JB, Swanson SJ, Jackson M, Whittern DN (1991) Revised NMR assignments for rapamycin. J Antibiot 44: 688-690

Mennigmann HD, Lange M (1986) Growth and differentiation of *Bacillus subtilis* under microgravity. Naturwissenschaften 73: 415-417

Mishra SK, Pierson DL (1992) Space flight effects on microorganisms. In: Lederberg J (ed) Encyclopedia of Microbiology Vol 4, Academic Press, San Diego pp 53-60

Moatti N, Lapchine L, Gasset G, Richoilley G, Templier J, Tixador R (1986) The "Antibio" experiment. Naturwissenschaften 73: 413-414

Paiva NL, Demain AL, Roberts MF (1991) Incorporation of acetate, propionate, and methionine into rapamycin by *Streptomyces hygroscopicus*. J Nat Prods 54: 167-177

Paiva NL, Demain AL, Roberts MF (1993a) The cyclohexane moiety of rapamycin is derived from shikimic acid in *Streptomyces hygroscopicus*. J Indust Microbiol 12: 423-428

Paiva NL, Demain AL, Roberts MF (1993b) The immediate precursor of the nitrogen-containing ring of rapamycin is free pipecolic acid. Enzyme Microb Technol 15: 581-585

Prewett TL, Goodwin TJ, Spaulding GR (1993) Three-dimensional modeling of T-24 human bladder carcinoma cell line: a new simulated microgravity culture vessel. J Tiss Cult Meth 15: 29-36

Reynolds KA, Demain AL (1997) Rapamycin, FK506 and ascomycin-related compounds. In: Strohl WR (ed) Biotechnology of Antibiotics. 2nd ed. Marcel Dekker, New York, pp 497-520

Sehgal SN, Molnar-Kimber K, Ocain TP, Weichman BM (1994) Rapamycin: A novel immunosuppressive macrolide. Med Res Rev 14: 1-22

Spizizen J, Isherwood JE, Taylor GR (1975) Effects of solar ultraviolet radiation on *Bacillus subtilis* spores and T-7 bacteriophage. Life Sci Space Res 13: 143-149

Tixador R, Richolilley G, Gasset G, Templeier J, Bes JC, Moatti N, Lapchine L (1985) Study of minimal inhibitory concentration of antibiotic on bacteria cultivated in vitro in space (cytos 2 experiment). Av Space Environ Med 56: 748-751

Vezina C, Kudelski A, Sehgal SN (1975) Rapamycin (AY-22,989), a new antifungal antibiotic. 1. Taxonomy of the producing streptomycete and isolation of the active principle. J Antibiot 28: 721-726

Wong GK, Griffith S, Kojima I, Demain AL (1998) Antifungal activities of rapamycin and its derivative, propylrapamycin, 32-desmethylrapamycin, and 32-desmethoxyrapamycin. J Antibiot 51: 487-491

Zhukov-Verezhnikov NN, Volkov MN, Maisky IN, Rybakov NI, Guberniev MA, Podoplelov II, Kulagin AN, Aniskin ED, Rybakova KD, Sharyi NI, Voronokova IP, Saxonov PP, Kopyev VY, Antipov VV, Kozlov VA, Parfyonov GP, Orlovsky VI (1971) Experiments with micro-organisms and human cell cultures in the Zond 5 and Zond 7 flights. In: Vishniac W (ed) COSPAR Life Sciences and Space Research vol 9, Akademie-Verlag, Berlin pp 99-103

Table 1. Inhibition of rapamycin production by growth in the RWB under SMG

| Condition | Growth DCW (mg/ml) | Rapamycin Production | |
|-----------|---------------------|------------|----------|
| | | volumetric (μg/ml) | specific (μg/mg DCW) |
| SMG | 0.46 | 1.5 | 4.0 |
| NG | 1.3 | 56 | 42 |

Table 2. Inhibition of rapamycin production by growth in SMG in the presence of beads

| Condition | Growth DCW (mg/ml) | Rapamycin Production | |
|---|---|---|---|
| | | volumetric (μg/ml) | specific (μg/mg DCW) |
| SMG | 1.7 | 8.4 | 5.1 |
| NG | 1.9 | 45 | 23 |

Table 3. Growth in SMG favors excretion of rapamycin into the medium

| Growth condition | Rapamycin (µg/ml) | Cellular rapamycin (%) | Supernatant rapamycin (%) |
|---|---|---|---|
| Unbaffled flasks | 339 | 56 | 44 |
| Baffled flasks | 357 | 62 | 38 |
| RWB in NG configuration | 52 | 54 | 46 |
| RWB in NG configuration containing 25 beads | 50 | 54 | 46 |
| RWB in SMG configuration | 2.3 | 34 | 66 |
| RWB in SMG configuration containing 25 beads | 11 | 40 | 60 |