Applied and Environmental Microbiology, Oct. 1997, p. 4090–4092 Vol. 63, No. 10
0099-2240/97/$04.00+0
Copyright © 1997, American Society for Microbiology

# Effect of Simulated Microgravity and Shear Stress on Microcin B17 Production by *Escherichia coli* and on Its Excretion into the Medium

A. FANG,[1] D. L. PIERSON,[2] D. W. KOENIG,[3] S. K. MISHRA,[3]† and A. L. DEMAIN[1]*

*Fermentation Microbiology Laboratory, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139,[1] and Johnson Space Center, National Aeronautics and Space Administration,[2] and KRUG Life Sciences, Inc.,[3] Houston, Texas 77058*

Received 14 March 1997/Accepted 16 July 1997

**Production of the antibacterial polypeptide microcin B17 (MccB17) by *Escherichia coli* ZK650 was inhibited by simulated microgravity. The site of MccB17 accumulation was found to be different, depending on whether the organism was grown in shaking flasks or in rotating bioreactors designed to establish a simulated microgravity environment. In flasks, the accumulation was cellular, but in the reactors, virtually all the microcin was found in the medium. The change from a cellular site to an extracellular one was apparently not a function of gravity, since extracellular production occurred in these bioreactors, irrespective of whether they were operated in the simulated microgravity or normal gravity mode. More probably, excretion is due to the much lower degree of shear stress in the bioreactors. Addition of even a single glass bead to the 50-ml medium volume in the bioreactor created enough shear to change the site of MccB17 accumulation from the medium to the cells.**

We have been interested in determining whether gravity affects microbial production of secondary metabolites (3, 4). In the present study, we examined the effect of simulated microgravity (SMG) on microcin B17 (MccB17) production by *Escherichia coli* ZK650 (5).

We recently reported that specific MccB17 production is positively related to growth rate and extent when oxygen limitation is used to decrease growth in a glucose-based chemically defined medium (5). On the other hand, specific production of MccB17 was negatively related to growth rate and extent when other nutritional limitations were imposed, in agreement with Connell et al. (1). Moreover, we found that glucose, glycerol, and acetate repressed MccB17 production but succinate did not. In this study, we report that MccB17 formation is markedly inhibited by SMG. We also show that its typical location, in or on the cells, is shifted to an extracellular site when the culture is grown in the rotating bioreactors, where shear stress is almost eliminated.

The media and analyses of growth and MccB17 production were as previously described (5). The high-aspect rotating vessels (HARVs), designated at the Johnson Space Center of the National Aeronautics and Space Administration to create conditions simulating microgravity (Synthecon Inc., Houston, Tex.), have been described in previous studies (3, 4). The seed medium was used in 250-ml unbaffled flasks at 25 ml per flask. The production medium was the same as the seed medium except for glucose which was held at 0.5 g/liter to avoid repression of MccB17 formation (5). Production medium was used at 190 ml per flask and incubated on a rotary shaker at 120 rpm for 48 h. The HARVs were positioned so that the axis of rotation is perpendicular to the gravity vector or with the axis parallel to the gravity vector. The former position provides an environment that simulates microgravity (SMG). On the other hand, the latter position provides essentially a normal-gravity (NG) environment. The HARVs were prepared for fermentation and incubated as previously described (4) except that 4% (vol/vol) of a seed culture was used. To assay cellular MccB17, duplicate 0.5-ml samples of whole broth were placed into two microcentrifuge tubes and centrifuged at 15,000 × $g$ for 6 min. The supernatant fluids were saved for assay of extracellular MccB17, if desired, or for assay of total (cellular plus extracellular) MccB17 (see below). Acetic acid (0.1 ml of a 100 mM solution containing 1 mM EDTA) was added to the pellets, and the cells were extracted at 100°C for 10 min. After the suspensions were cooled, they were centrifuged for 6 min, and the extracts, considered to be fivefold concentrates of the original broths, were used for bioassay. To assay total (cellular plus extracellular) MccB17, the broth supernatant fluids mentioned above (~0.5 ml) were added to the extracted cell suspension and centrifuged for 6 min and the supernatant fluids were used for bioassay. These supernatant fluids were considered to be equivalent to 20% diluted broths.

**Inhibition of MccB17 production by SMG.** The initial experiments were done when only a single HARV was available to establish SMG. For NG controls, we used 250-ml unbaffled Erlenmeyer flasks containing 190 ml of medium (5). We found that MccB17 was produced in SMG, but production was only about 30% of that in NG.

The modified HARV and flask systems used above for the SMG versus NG environments, respectively, differ not only in gravity but also in vessel geometry and shear stress. The opportunity to eliminate the latter two variables presented itself when two identical HARVs became available for our use. MccB17 production was found to be inhibited by SMG, whereas growth rate and extent were stimulated (Fig. 1).

**Growth in HARVs changes MccB17 production from its cellular location to an extracellular location.** Up to this point in our investigation, all MccB17 assays had been done with cell extracts rather than culture supernatant fluids, since Dav-

* Corresponding author. Mailing address: Fermentation Microbiology Laboratory, Department of Biology, Massachusetts Institute of Technology, 68-223, Cambridge, MA 02139. Phone: (617) 253-1711. Fax: (617) 253-8550. E-mail: demain@mit.edu.

† Present address: Xechem, New Brunswick, NJ 08901-3279.

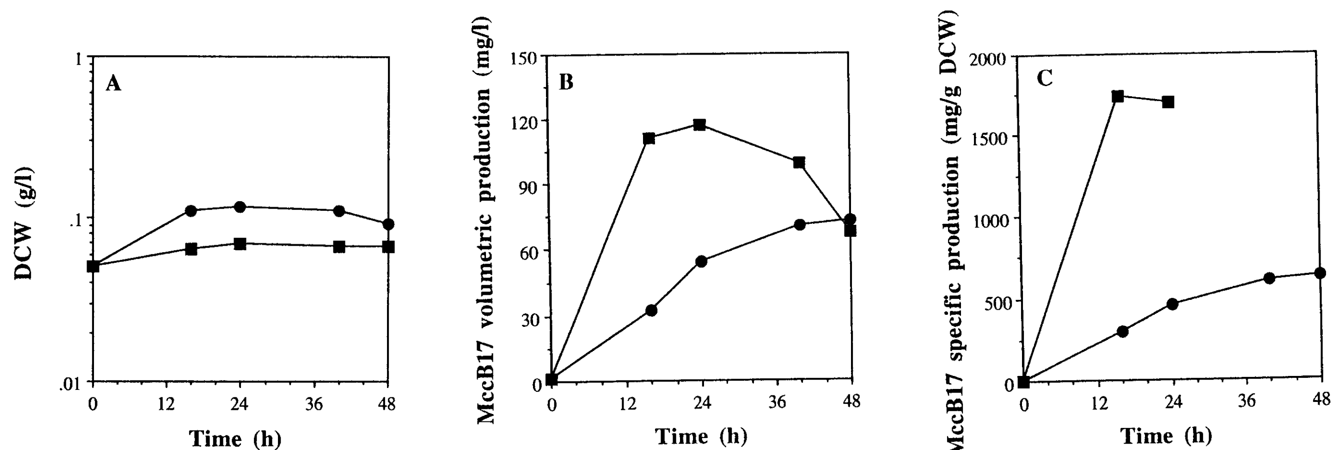


FIG. 1. Inhibition of MccB17 production by SMG. SMG (●) and NG (■) were used. Fermentations were done in identical HARVs. Glucose concentration was 0.5 g/liter. Growth (A) volumetric production of MccB17 (B), and specific production of MccB17 (C) are shown. DCW, dry weight of cells.

agnino et al. (2) and our earlier work (5) had shown virtually no excretion or leakage of MccB17 from the outer membrane into the medium. We decided to verify this observation with cultures grown for 48 h in SMG in the modified HARV and were surprised to discover that almost all the MccB17 was in the external broth and almost none was in or on the cells. As expected, MccB17 in the flasks under NG was almost completely associated with the cells. Total production (in cell extract plus culture supernatant fluid) under SMG in the modified HARV was still much less than under NG in flasks, confirming our preliminary conclusion that SMG inhibits MccB17 production. In the remaining experiments, all assays were done with samples containing both cell extract and supernatant fluid.

We next used the two HARVs to determine whether the change of MccB17 from a cellular location in flasks to an extracellular one in the modified HARV was a gravity effect. We also included flasks with both high (25 ml of medium/250-ml flask) and low (190 ml of medium/250-ml flask) aeration. Table 1 shows that the change in location was not a function of gravity, i.e., extracellular production was seen in the HARV in both SMG and NG modes of operation. The cellular location of MccB17 in flasks was unaffected by the level of aeration.

At this point, our tentative conclusion was that the change from cellular to extracellular production of MccB17 is due to the much lower shear stress characteristic of the HARVs. To examine this point, we added beads (Teflon PTFE Solid Balls [1/8 in. diameter]; Norton Performance Plastic Corp., Wayne, N.J.) to create shear stress in the HARV during growth and MccB17 production. We found that addition of even a single bead to the 50 ml of medium in the HARV under SMG changed the site of accumulation from 91% extracellular to 98% cellular (Table 2). Moreover, virtually no difference was seen between the effect of 1 bead versus that of 25 beads. Presumably, the shear stress created by a single bead is enough to bring about cellular localization of the MccB17. Interestingly, adding beads to the HARV rotated in the NG mode had only a partial effect on the change from extracellular to cellular production (Table 2). The reason for this is that the beads do not move freely in the NG mode of operation, i.e., they remain at the periphery of the reactor. In contrast, the beads continually move freely throughout the liquid in SMG.

The results described here show that production of MccB17 by *E. coli* is markedly inhibited by SMG although growth is stimulated. The dramatic change in the site of MccB17 accumulation from the cells (as seen in flasks) to the extracellular fluid (first seen when *E. coli* ZK650 was grown in the modified HARV) was totally unexpected. Later work in the duplicate HARVs showed that the change did not depend on SMG. Excretion into the medium seems to be a function of the extremely low shear stress characteristic of the HARV environment. Determining whether this phenomenon is specific for MccB17 or is more general in nature will be critical. If the latter is the case, then the use of HARVs could hold major biotechnological promise for excretion of normally cellular

TABLE 1. Site of MccB17 accumulation in HARVs conducted under SMG and NG and in flasks[a]

| Gravity status | Vessel | % of MccB17 in: | |
|---|---|---|---|
| | | Cells | Culture supernatant |
| SMG | HARV | 7.7 | 92.3 |
| NG | HARV | 4.6 | 95.4 |
| NG | 250-ml flask with 190 ml of medium | 97.8 | 2.2 |
| NG | 250-ml flask with 25 ml of medium | 94.2 | 5.8 |

[a] Medium contained 0.5 g of glucose per liter. All samples are from broth taken at 48 h.

TABLE 2. Effect of beads on site of MccB17 accumulation in HARVs conducted under SMG and NG[a]

| Gravity status | No. of beads | % of MccB17 in: | |
|---|---|---|---|
| | | Cells | Culture supernatant |
| SMG | 0 | 8.7 | 91.3 |
| | 1 | 97.9 | 2.1 |
| | 5 | 98.0 | 2.0 |
| | 15 | 97.2 | 2.8 |
| | 25 | 97.2 | 2.8 |
| NG | 0 | 9.1 | 90.9 |
| | 25 | 41.4 | 58.6 |

[a] Medium contained 0.5 g of glucose per liter. All samples are from broth taken at 48 h.

products into the medium, possibly enhancing production and lowering the costs of downstream processing.

The work was supported by National Aeronautics and Space Administration grant no. NAG 9-602.

We appreciate the editorial help of Christine Wogan with preparation of the manuscript.

## REFERENCES

1. **Connell, N., Z. Han, F. Moreno, and R. Kolter.** 1987. An *E. coli* promoter induced by the cessation of growth. Mol. Microbiol. **1:**195–201.
2. **Davagnino, J., M. Herrero, D. Furlong, F. Moreno, and R. Kolter.** 1986. The DNA replication inhibitor microcin B17 is a forty-three amino acid protein containing sixty percent glycine. Proteins **1:**230–238.
3. **Fang, A., D. L. Pierson, S. K. Mishra, D. W. Koenig, and A. L. Demain.** 1997. Secondary metabolism in simulated microgravity: β-lactam production by *Streptomyces clavuligerus*. J. Ind. Microbiol. Biotechnol. **18:**22–25.
4. **Fang, A., D. L. Pierson, S. K. Mishra, D. W. Koenig, and A. L. Demain.** 1997. Gramicidin S production by *Bacillus brevis* in simulated microgravity. Curr. Microbiol. **34:**199–204.
5. **Fang, A., and A. L. Demain.** 1997. Influence of aeration and carbon source on production of microcin B17 by *Escherichia coli* ZK650. Appl. Microbiol. Biotechnol. **47:**547–553.