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Induction of microcin B17 formation in *Escherichia coli* ZK650 by limitation of oxygen and glucose is independent of glucose consumption rate

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We examined the consumption of glucose from the media in which *Escherichia coli* ZK650 was grown. This organism, which produces the polypeptide antibiotic microcin B17 best under conditions of limiting supplies of glucose and air, was grown with a low level of glucose (0.5 mg/ml) as well as a high level (5.0 mg/ml) under both high and low aeration. Glucose consumption rates were virtually identical under both high and low aeration. Thus, glucose consumption rate is not a regulating factor in microcin B17 formation. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 341–344.

Keywords: Microcin B17; *Escherichia coli*; glucose; oxygen

Introduction

Air supply has a major effect on production of microcin B17 (MccB17) by *Escherichia coli* ZK650 [3] In shaken flasks receiving a high level of aeration (25 ml medium/250 ml Erlenmeyer flask), production is poor. When a large volume of medium is used in the same-size flask (170–190 ml/250 ml flask), mild air limitation occurs, resulting in a decrease in growth rate and growth extent. Under this condition, production of MccB17 is at its peak level. When higher levels of medium are used (200–230 ml/flask), causing severe air limitation, growth and production decrease markedly [3]. At the optimum aeration level, a high glucose level (5 mg/ml) markedly represses production. A low glucose concentration (0.5 mg/ml) is required to support effective production of MccB17. Thus, the best conditions for MccB17 production are mild air limitation, where growth rate and growth extent have just begun to decline, and the presence of limiting levels of carbon source. In the present study, we wanted to determine whether the marked difference in production at low *versus* high aeration is related to the glucose consumption rate.

Materials and methods

Medium

The seed and production medium was M63 plus glucose and ampicillin [6,8]. It contained (g/l) glucose 0.5 or 5, $(NH_4)_2SO_4$ 8, KH_2PO_4 12, K_2HPO_4 28, $MgSO_4{\cdot}7H_2O$ 0.25, thiamine 0.001 and ampicillin 0.15. Glucose was autoclaved separately from the inorganic salts of the medium. Thiamine was sterilized by filtration and ampicillin was sterilized by dissolving it in 40% ethanol. The rest of the medium were placed in 250-ml unbaffled flasks at 25 or 190 ml/flask and autoclaved.

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Strains

The production culture was *E. coli* ZK650, an *E. coli* K12 derivative [ZK4(F^- *araD139* Δ*lacU169 rpsL relA thiA recA56*)+pPY113] that contains the high-copy-number plasmid pPY113 [11] for MccB17 production. This plasmid is derived from pBR322 and contains *mcbABCDEFG* genes for production plus genes for resistance to ampicillin and tetracycline. The flasks were incubated on the shaker at 120 rpm at 37°C and bioassayed at various times over a period of 48 h. The bioassay strain ZK4 [11] is the same as ZK650, but lacks the plasmid.

Both stock cultures were prepared in Luria–Bertani (LB) broth containing Bactotryptone (Difco Laboratories, Detroit, MI) 10 g/l, Bacto yeast extract (Difco) 5 g/l and NaCl 10 g/l. The medium (25 ml) was used in 250-ml unbaffled conical flasks. Cultures were shaken at 200 rpm for 24 h at 37°C. The cells were stored at −20°C in 30% (v/v) glycerol as stock cell suspensions. Seed cultures were incubated at 37°C on a rotary shaker (5 cm) at 200 rpm for 20–24 h. Production cultures were inoculated with 4% (v/v) seed culture and incubated on a rotary shaker (5 cm diameter orbit) at 120 rpm for up to 40 h.

Analyses

Samples were collected from the flasks and growth was determined by absorbance by using a Klett Summerson colorimeter (Klett Manufacturing, New York, NY) with the red filter. The fermentation broths were diluted with distilled water and the turbidity was determined in the range of 50–150 Klett units. A dry cell weight of 1 g/l is equivalent to 270 Klett units.

To assay microcin B17, it must be extracted from the cell envelope. Duplicate 0.5-ml samples of whole broth were placed in two microcentrifuge tubes. Acetic acid (0.5 ml of 100 mM) containing 1 mM EDTA was added and the cells were extracted at 100°C for 10 min. After cooling them, the suspensions were centrifuged for 6 min and the supernatant fluids (considered a 1:1 dilution of the broths) were used for bioassay.

The production of MccB17 was determined by the agar plate disk diffusion assay with the assay strain ZK4 seeded at 100 μl/100

ml agar; the agar concentration was 8 g/l. Paper disks were saturated with 20 μl samples and placed on the surface of the seeded agar. Bioassay plates were placed at 4°C for 24 h to allow diffusion of MccB17 from the paper disks into the agar and then incubated for 14–16 h at 37°C. The diffusion step markedly increases the size of the clear inhibitory zones that result. A purified sample of MccB17 served as assay standard, but since it was not completely pure, we present antibiotic activity as units. One unit gives the same zone as one microgram of the standard. The agar zone assay was calibrated and used through the range tested.

Glucose was determined by using the YSI 2700 SELECT Biochemistry Analyzer (YSI, Yellow Springs, OH).

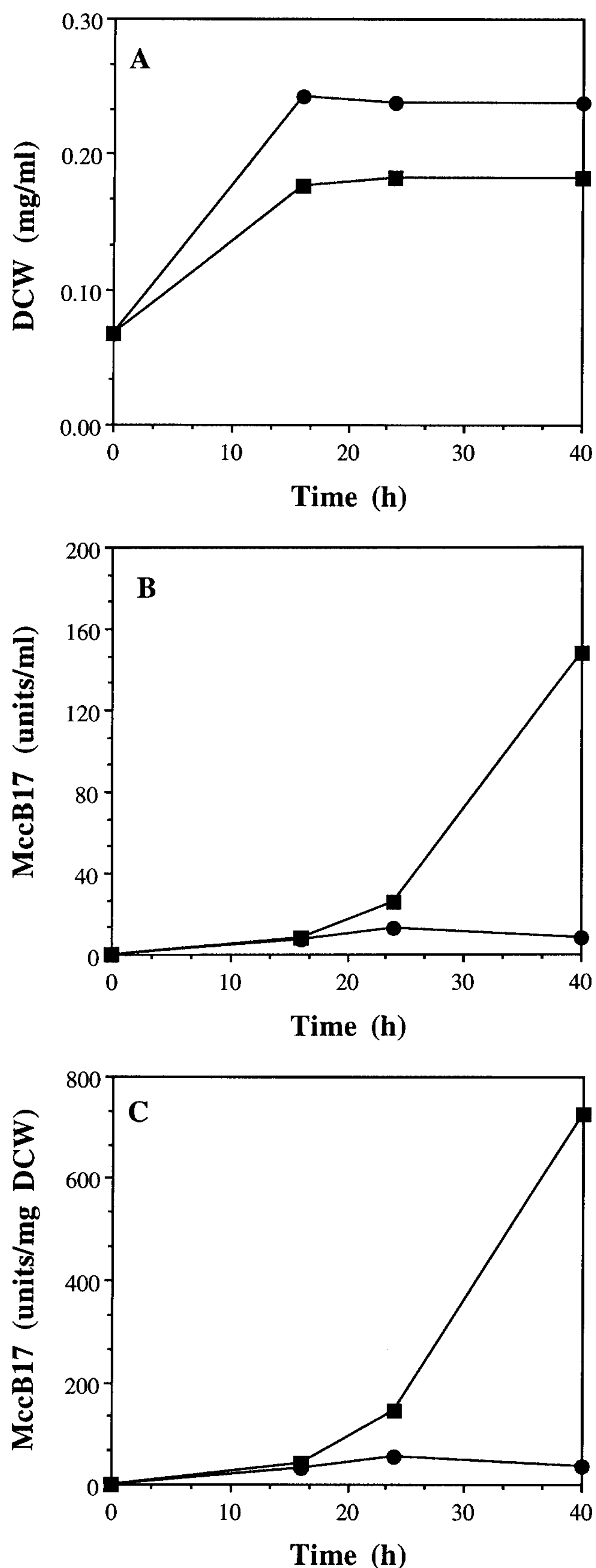

Figure 1 Growth and production of MccB17 at different aeration levels using a low (0.5 mg/ml) glucose concentration. (A) Growth as dry cell weight (DCW). (B) Volumetric MccB17 production. (C) Specific MccB17 production. (●) High aeration level. (■) Low aeration level.

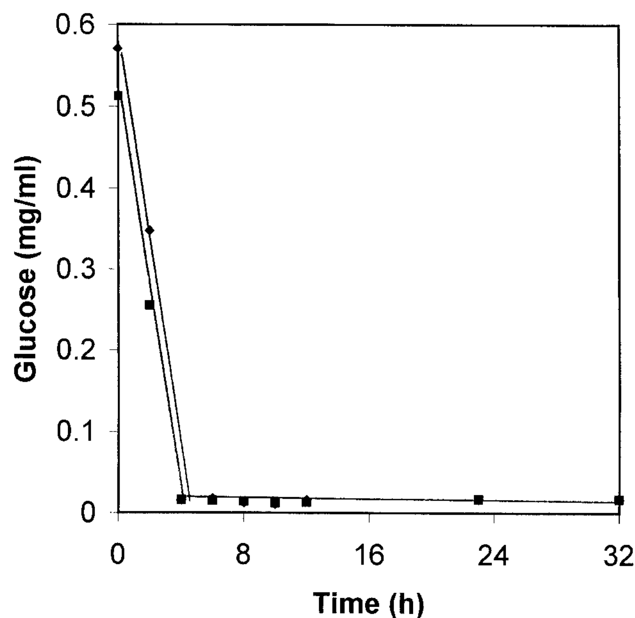

Figure 2 Glucose consumption at different levels of aeration using a low glucose concentration (0.5 mg/ml). (◆) High aeration. (■) Low aeration.

Results and discussion

Low glucose concentration

The initial experiments were done using a low glucose concentration, 0.5 mg/ml, previously found best for MccB17 production [3]. Growth and production are shown in Figure 1. It is clear that with high aeration (25 ml medium in a 250-ml shaken Erlenmeyer flask), growth was higher than at low aeration (190 ml medium/250 ml flask), but production of MccB17 was poorer. These new data agree with our previous published findings [3]. We next studied glucose consumption. Figure 2 shows that glucose was consumed under both conditions by 4 h. The rate of consumption of glucose was very similar under high or low aeration and thus could not have been responsible for the major difference in MccB17 production. We also determined growth rates and found that the growth rate was much higher under higher aeration (doubling time, d_t=156 min) than under low aeration (d_t=528 min). Thus, it was more likely that MccB17 production was controlled by growth rate rather than by the rate of glucose consumption.

High glucose concentration

Earlier, we reported that glucose at 5 mg/ml is very repressive to MccB17 formation under otherwise optimal conditions, i.e., under low aeration [3]. We also reported that high glucose was much more repressive to production under low aeration than under high aeration and that it had an opposite effect on growth, i.e., high glucose stimulated growth at high aeration much more than it did at low aeration, but no data were presented. Such relationships can be seen in Figure 3, which presents data obtained during the present work. High glucose under low aeration completely repressed MccB17 production while stimulating growth by about 70%. On the other hand, high glucose under high aeration had only a slight negative effect on specific MccB17 production while exerting a 400% increase in growth. It also is of interest that the rates of glucose utilization with high glucose are quite similar under the two aeration conditions (Figure 4), a situation seen earlier at the low glucose concentration (Figure 2). With regard to growth rates, examination of doubling times in Table 1 reveals that all the conditions resulting in short doubling times exhibited no or poor MccB17 production. Only the condition featuring low aeration and low glucose supported a high level of MccB17 production.

The present studies show that the marked positive effect of low aeration and low glucose concentration on production of MccB17 is not related to the rate of glucose consumption. Instead, good production was dependent upon (i) low aeration, (ii) low glucose supply and (iii) low growth rate. This suggests that stress, occurring as a result of both carbon and air starvation, induces formation of the *E. coli* antibiotic. It is understandable that in nature, stress would signal microorganisms to prepare themselves for hard times and start producing chemical weapons (e.g., antibiotics) as well as resistant or easily dispersed morphological forms of the culture (e.g., endospores or conidia, respectively). Since *E. coli* does not produce endospores or conidia, it must depend on antibiotic production.

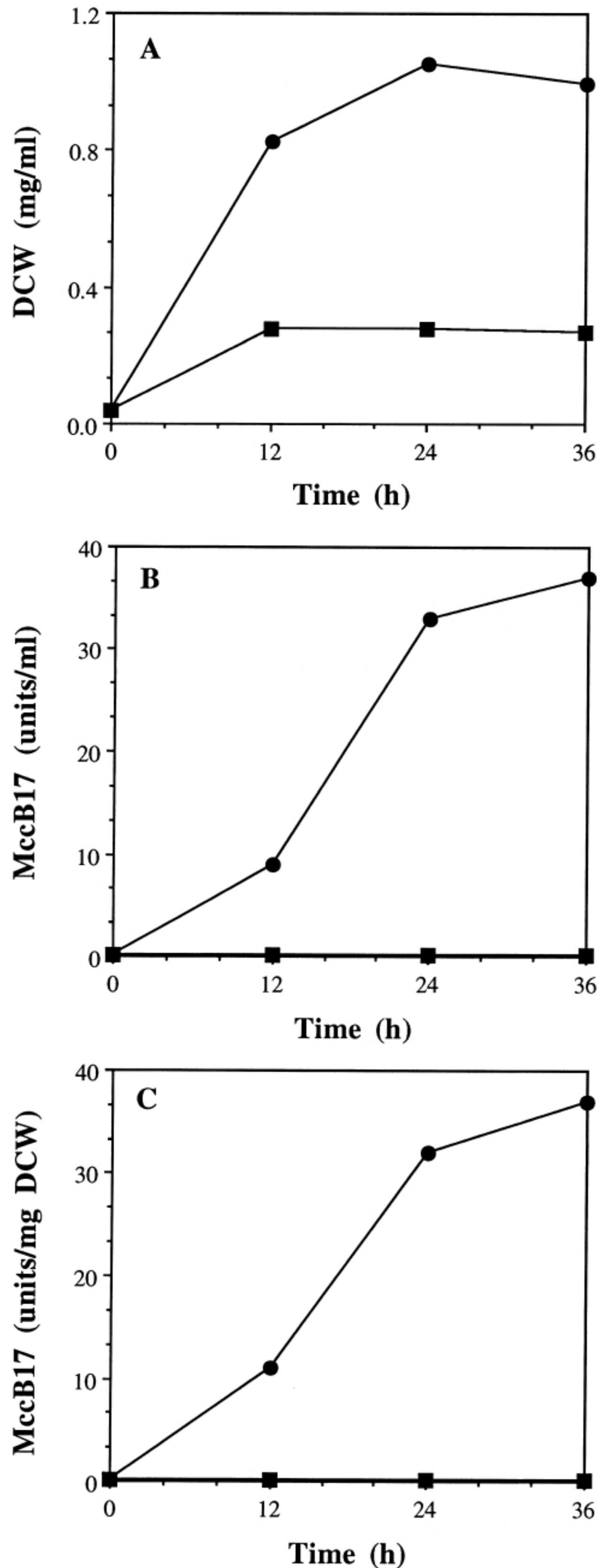

Figure 3 Growth and production of MccB17 at different aeration levels using a high (5 mg/ml) glucose concentration. (A) Growth as dry cell weight (DCW). (B) Volumetric MccB17 production. (C) Specific MccB17 production. (●) High aeration level. (■) Low aeration level.

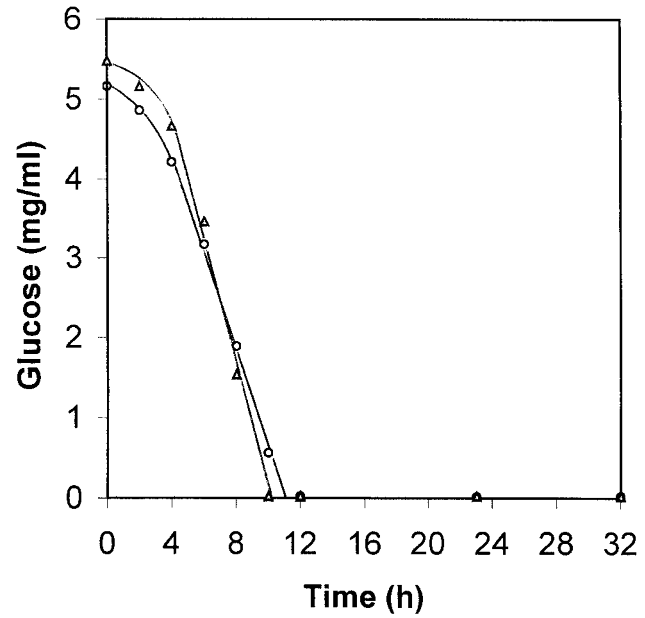

Figure 4 Glucose consumption at different levels of aeration using a high glucose concentration (5 mg/ml). (△) High aeration. (○) Low aeration.

Table 1 Effect of glucose concentration and aeration level on growth, glucose consumption and MccB17 production*

Glucose level	Aeration level	d_t (min)	Maximum DCW (mg/ml)	Maximum volumetric MccB17 (u/ml)	Maximum specific MccB17 (u/mg DCW)	Glucose consumption rate (mg/ml/h)
Low	Low	528	0.17	148	725	0.16
High	Low	180	0.29	0	0	0.61
Low	High	156	0.23	13	55	0.13
High	High	138	1.11	37	37	0.81

*Flasks were sampled and assayed at 0, 2, 4, 6, 8, 10, 12, 23 and 32 h for growth rate [doubling time (d_t)] and glucose consumption assays. Additional later samplings up to 40 h were done for maximum DCW and MccB17 assays.

Although basic research on stress, and its sigma factors and proteins, is currently at a high level [7,10], little is known about the induction of antibiotic production by stress factors. However, a few studies, whose findings are as follows, have been published: (i) ethanol and heat shock induce jadomycin formation by *Streptomyces venezuelae* ISP5230 [2]; (ii) growth with ethanol increases the bioconversion of penicillins into cephalosporins by resting cells of *S. clavuligerus* [4]; (iii) antibiotic formation by *Pseudomonas fluorescens* S272 is promoted by high ethanol, NaCl and heat shock [9]; (iv) oxygen limitation induces production of erythromycin and a novel pigmented secondary metabolite by *Saccharopolyspora erythrea* NRRL 2338 [1]. Whether these findings are at all related to each other, or to MccB17 production, remains to be elucidated. Recently, we have found that shear stress, but not ethanol stress, enhances MccB17 formation [5].

Acknowledgements

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