

## Effect of Monensin on Growth and Methanogenesis of *Methanobacterium formicicum*

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**Monensin inhibited methanogenesis from formate but not from H<sub>2</sub>-CO<sub>2</sub> by resting-cell suspensions of *Methanobacterium formicicum*. The antibiotic severely inhibited growth on formate. The lag phase of H<sub>2</sub>-CO<sub>2</sub>-grown cultures was prolonged by monensin, but these cultures recovered from the initial inhibition. The recovery did not result from the development of a monensin-resistant population or inactivation of the antibiotic.**

Monensin is an ionophore which is added to ruminant diets to improve the efficiency of feed utilization (8, 9). Dietary monensin also inhibits methanogenesis from cattle wastes (12, 13). The antibiotic transiently inhibits methanogenesis from H<sub>2</sub>-CO<sub>2</sub> (3) and H<sub>2</sub> uptake (4) by pure cultures. The growth of methanogenic organisms is also sensitive to monensin when assayed by the agar diffusion test (5). The objective of this study was to characterize the effect of monensin on both growth and methanogenesis from both H<sub>2</sub>-CO<sub>2</sub> and formate by *Methanobacterium formicicum* to obtain a better understanding of monensin inhibition of methanogenic bacteria.

*M. formicicum* JF-1 (DSM 2639), isolated from sewage sludge, is described elsewhere (10). Basal medium contained (in final [percent] compositions by weight): K<sub>2</sub>HPO<sub>4</sub>, 0.0225; KH<sub>2</sub>PO<sub>4</sub>, 0.0225; NH<sub>4</sub>Cl, 0.143; NaCl, 0.045; MgSO<sub>4</sub>, 0.0045; CaCl<sub>2</sub> · 2H<sub>2</sub>O, 0.006; FeNH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub>, 0.001; resazurin, 0.0001; Na<sub>2</sub>SeO<sub>3</sub>, 0.00002; Antifoam C (Sigma Chemical Co., St. Louis, Mo.), 0.017; sodium acetate, 0.2; cysteine hydrochloride, 0.025; and Na<sub>2</sub>S · 9H<sub>2</sub>O, 0.025. Antifoam C is an emulsion of silicon polymer which suppresses foam formation. Vitamin and trace mineral solutions (14) were each added to a final concentration of 1% (vol/vol). The trace mineral solution was amended with 0.0025% (wt/vol) sodium molybdate and 0.0027% (wt/vol) nickelous sulfate. Medium for formate-grown tube cultures was basal medium supplemented with 0.4% (wt/vol) Na<sub>2</sub>CO<sub>3</sub> and 0.6% (wt/vol) sodium formate (final pH, 7.2). Basal medium supplemented with 0.4% (wt/vol) Na<sub>2</sub>CO<sub>3</sub> (final pH, 7.2) was used for cultivation with H<sub>2</sub>-CO<sub>2</sub>. Formate-grown and H<sub>2</sub>-CO<sub>2</sub>-grown tube cultures were pressurized with 150 kPa N<sub>2</sub>-CO<sub>2</sub> (80:20) and 150 kPa H<sub>2</sub>-CO<sub>2</sub> (80:20) as previously described (1).

Anaerobic shake flask cultures (2) sparged with H<sub>2</sub>-CO<sub>2</sub> (80:20) (absorbance at 550 nm, 0.6) were used for inocula and cell material for resting-cell studies. The medium was basal medium supplemented with 0.6% (wt/vol) formate and 0.3% (wt/vol) Na<sub>2</sub>CO<sub>3</sub> (final pH, 7.5). Cell dry weight was determined by the filtration of cultures through tared 0.22- $\mu$ m membrane filters (Millipore Corp., Bedford, Mass.). The filters were then dried to a constant weight at 95°C. One unit of absorbance at 550 nm corresponded to 0.44 g (dry weight) per liter.

Monensin-sodium (Eli Lilly & Co., Greenfield, Ind.) was dissolved in diethyl ether which was added to tubes and

evaporated before addition of the medium (9.8 ml). The inoculum was 0.2 ml of shake flask culture. Tubes were shaken at 37°C in a slanted position. Cultures grown with H<sub>2</sub>-CO<sub>2</sub> were repressurized daily. A Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.) was used to follow growth at 550 nm.

Methane was determined with a silica gel column in a gas chromatograph equipped with a flame ionization detector (11). Determination of H<sub>2</sub> was done with a gas chromatograph equipped with a thermal conductivity detector (10). Gas pressure in culture tubes was determined with a 10-ml glass syringe by displacement of the barrel after insertion of the syringe needle through the stopper.

Cells for resting-cell studies were washed twice in oxygen-free 50 mM sodium phosphate buffer (pH 7.5) which contained 0.0001% (wt/vol) resazurin and 5 mM cysteine hydrochloride. Reactions were performed in 5-ml serum vials sealed with butyl rubber serum stoppers (Bellco Glass, Inc., Vineland, N.J.). The final reaction mixture (1 ml) contained 45 mM sodium phosphate buffer (pH 7.5), 0.0001% (wt/vol) resazurin, 4.5 mM cysteine hydrochloride, 100 mM NaCl, and 0.20 mg of washed cells (dry weight) per ml. All manipulations were done under an atmosphere of oxygen-free N<sub>2</sub> (12). The N<sub>2</sub> atmosphere was replaced by H<sub>2</sub>-CO<sub>2</sub> (80:20) to initiate methanogenesis from H<sub>2</sub>-CO<sub>2</sub>. When formate was the substrate, the reaction mixture also contained 40 mM sodium formate. Incubation was at 37°C in a water bath shaker.

The rate of methanogenesis (61 nmol min<sup>-1</sup> mg<sup>-1</sup> [dry weight]) from H<sub>2</sub>-CO<sub>2</sub> by resting-cell suspensions of H<sub>2</sub>-CO<sub>2</sub>-grown *M. formicicum* was not inhibited by 40 or 80  $\mu$ g of monensin per ml. However, the antibiotic prolonged the lag phase and decreased the growth rate in pressurized tubes (Fig. 1). The effect on growth was concentration dependent. The maximum growth rate (0.014 h<sup>-1</sup>) with 40  $\mu$ g of monensin per ml was 61% of the control without monensin (0.023 h<sup>-1</sup>). In the presence of monensin, the lag in methanogenesis was the same as that of growth, but after 4 days the rates at all concentrations were the same as that of the control (0.13 mmol of methane per day) without the antibiotic (data not shown). This was consistent with previous reports (3, 4). Our results indicate that monensin partially uncoupled growth (ATP synthesis) from methanogenesis. The proton motive force is thought to drive ATP synthesis in methanogenic bacteria (7), and monensin collapses an imposed pH gradient (inside alkaline) and decreases intracellular ATP concentrations in *Methanobacterium bryantii* (6). This property of monensin is consistent with our results.

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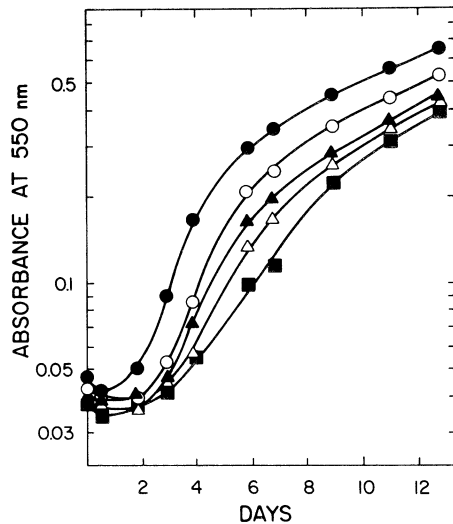


FIG. 1. Effect of monensin on growth of *M. formicicum* with  $H_2$ - $CO_2$  (80:20) in pressurized tubes. Monensin was added at 0 (●), 2.5 (○), 5 (▲), 20 (△), and 40 (■)  $\mu\text{g/ml}$ . Values are the mean of three replicate cultures.

The cultures eventually recovered from the initial inhibition of growth, but the recovery was incomplete at high concentrations of the antibiotic (Fig. 1). The possibility that this recovery resulted from inactivation of monensin during growth was investigated. Cells were removed by anoxic centrifugation from the spent media of 13-day-pressurized tube cultures that contained monensin (10  $\mu\text{g/ml}$ ). This spent medium was diluted with an equal volume of fresh medium without the antibiotic. Growth and methanogenesis from  $H_2$ - $CO_2$  in this diluted spent medium (5  $\mu\text{g}$  of monensin per ml) were compared with control cultures grown in diluted spent medium without monensin. The resulting pattern of inhibition was the same as that shown in Fig. 1 for cultures grown in fresh medium with an equivalent amount of the antibiotic (5  $\mu\text{g/ml}$ ). These results suggest that growing cultures did not

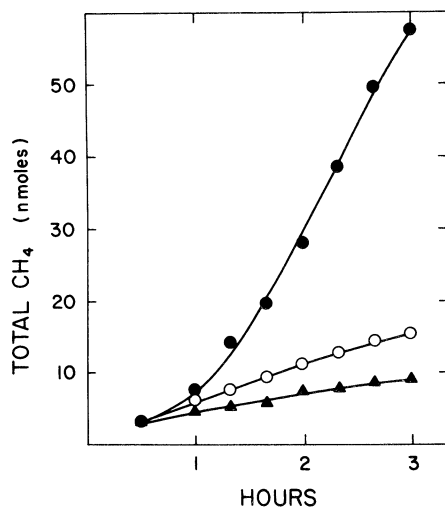


FIG. 2. Effect of monensin on methanogenesis from formate by resting-cell suspensions of formate-grown *M. formicicum*. Monensin was added at 0 (●), 1 (○), and 2.5 (▲)  $\mu\text{g/ml}$ . Values are means of four replicate reaction vials.

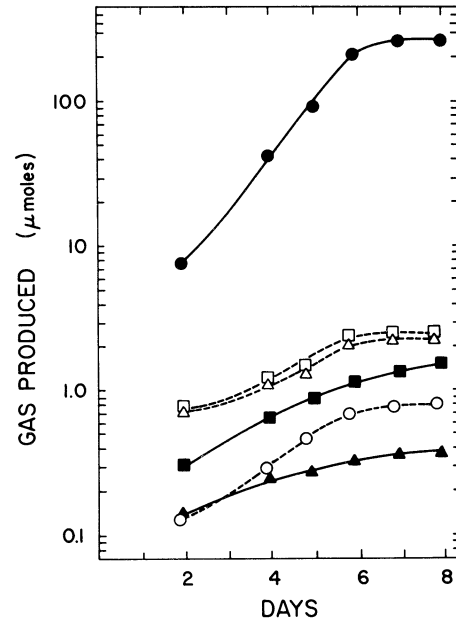


FIG. 3. Effect of monensin on methanogenesis and  $H_2$  production in formate-grown tube cultures of *M. formicicum* pressurized with  $N_2$ - $CO_2$  (80:20). Methanogenesis was with monensin added at 0 (●), 2.5 (■), and 5 (▲)  $\mu\text{g/ml}$ .  $H_2$  production was with monensin added at 0 (○), 2.5 (△), and 5 (□)  $\mu\text{g/ml}$ . Values shown are the mean of three replicate cultures.

inactivate the monensin. When cultures grown in the presence of monensin (2.5  $\mu\text{g/ml}$ ) were used as inocula, the lag in growth was similar to that shown in Fig. 1 (2.5  $\mu\text{g/ml}$ ). These results suggest that a monensin-resistant population did not develop with 2.5  $\mu\text{g}$  of antibiotic per ml.

The rate of methanogenesis from formate by resting-cell suspensions of formate-grown cells was severely inhibited by only 1  $\mu\text{g}$  of monensin per ml (Fig. 2). The antibiotic (2.5  $\mu\text{g/ml}$ ) also inhibited methanogenesis in formate-grown cultures (Fig. 3); growth followed the same pattern as methanogenesis (data not shown). Formate-grown cultures with monensin produced more  $H_2$  than methane, whereas cultures without the antibiotic produced much smaller amounts of  $H_2$  than methane (Fig. 3).

The inhibition of growth and methanogenesis was considerably greater in formate-grown than in  $H_2$ - $CO_2$ -grown cultures. The inhibition of methanogenesis from formate, but not from  $H_2$ - $CO_2$ , in resting-cell suspensions suggests that the greater inhibition of formate-dependent growth resulted from the inhibition of methanogenesis. The greater production of  $H_2$  from formate in the presence of monensin (Fig. 3) indicates that  $CO_2$  reduction to methane was inhibited; however, other factors such as inhibition of formate transport could have contributed.

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