

Uncoupling of Methanogenesis from Growth of *Methanosarcina barkeri* by Phosphate Limitation

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Received 20 May 1985/Accepted 13 August 1985

Production of methane by *Methanosarcina barkeri* from H_2 - CO_2 was studied in fed-batch culture under phosphate-limiting conditions. A transition in the kinetics of methanogenesis from an exponentially increasing rate to a constant rate was due to depletion of phosphate from the medium. The period of exponentially increasing rate of methanogenesis was extended by increasing the initial concentration of phosphate in the medium. Addition of phosphate during the constant period changed the kinetics to an exponentially increasing rate of methanogenesis, indicating the reversibility of phosphate depletion. The relation between methanogenesis and growth of *M. barkeri* was investigated by measuring the incorporation of phosphorus, supplied as $KH_2^{32}PO_4$, in the medium. At a low (1 μM) initial concentration of phosphate in the medium and during the constant period of methanogenesis, there was no net cell growth. At a higher (10 μM) initial concentration of phosphate, cell growth proceeded linearly with time after phosphate had been removed from the medium by uptake into cells.

Production of methane by the methanogenic bacteria is a means of deriving energy (4, 7). Utilization of the energy-yielding substrate can be uncoupled from growth of some eubacteria by phosphate limitation (5, 18), and this coupling can be controlled reversibly by the availability of phosphate. The growth of one methanogenic bacterium, *Methanobacterium thermoautotrophicum*, under phosphate-limiting conditions has been described previously (17). In the absence of added phosphate, exponential growth of *M. thermoautotrophicum* did not occur, although linear growth kinetics were demonstrated for a short period and evidence was presented that cyclic-2,3-diphosphoglycerate, a phosphate storage compound, was utilized during the phosphate starvation (17). The coupling between methanogenesis and cell growth was not investigated in that study.

Reports have indicated that the coupling of cell growth to methanogenesis can be disrupted (7, 15, 16), although little detailed attention has been paid to this phenomenon. In a fed-batch, mixed methanogenic, calcium acetate enrichment culture it was shown that a transition from an exponentially increasing rate of methanogenesis to a constant rate occurred before acetate was depleted (10). This transition in kinetics could be explained by uncoupling of growth from methanogenesis (8, 10). Control of the coupling could be exploited to advantage in anaerobic digestion (8, 10), and, accordingly, the effect of phosphate limitation on a methanogen in pure culture was studied. *Methanosarcina barkeri* was chosen because it was the most abundant bacterium in the calcium acetate enrichment culture (8), and strain Fusaro was used because its nutritional requirements are well studied and can be met in defined medium (14).

MATERIALS AND METHODS

Methanogenic bacterium and culture. *M. barkeri* Fusaro was obtained from the Deutsche Sammlung von Mikroorganismen (DSM 804). Phosphate-free defined medium (pH 6.8) contained (per liter): sodium acetate, 2 g; NaCl, 0.9 g; $(NH_4)_2SO_4$, 0.9 g; $CaCl_2 \cdot 6H_2O$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; Na_2CO_3 , 1.2 g; resazurin, 1 mg;

and trace mineral solution (8). The medium was dispensed (4.5 or 400 ml) under N_2 - CO_2 (4:1 vol/vol) from which O_2 had been removed by passage over copper turnings at 300°C, and it was then autoclaved. Before use folic acid (5 μM) and riboflavin (5 μM) were added from stock solutions, and the medium was reduced by the addition of (per liter) cysteine hydrochloride (0.5 g) and $Na_2S \cdot 9H_2O$ (0.5 g). KH_2PO_4 was added from stock solutions as required. The gaseous headspace in either tubes (containing 4.5 ml of medium) or modified Duran bottles (containing 400 ml of medium) was replaced with H_2 - CO_2 (4:1 vol/vol) to a pressure of 2 atm (ca. 202.6 kPa), and cultures were incubated at 37°C while shaken at 200 rpm in an orbital shaker (New Brunswick Scientific Co., Inc., Edison, N.J.).

Incorporation of ^{32}P . $KH_2^{32}PO_4$ was obtained from Amersham International Ltd. $KH_2^{32}PO_4$ (100 μCi ; 3.7 MBq) was added to 400 ml of medium containing either no or 10 μM unlabeled phosphate to give final phosphate concentrations of 1.25 and 11.25 μM , respectively. At appropriate times 1-ml samples of culture were removed and stored frozen for later use in an enzyme-linked immunosorbent assay (ELISA). A 1-ml sample of culture was centrifuged ($9,980 \times g$, 5 min) in an Eppendorf model 5414 centrifuge. The supernatant was removed, and the cell pellets were washed three times in phosphate-buffered saline (pH 7.4) and then resuspended in 1 ml of phosphate-buffered saline. Radioactivity was estimated in all samples by Cerenkov counting (6) in a Phillips PW4700 liquid scintillation counter. The counting efficiency was 56%.

Other assays. The amount of *M. barkeri* present in samples was estimated in terms of protein by ELISA (1). Samples were diluted $\times 20$, and cells were broken by passage through a French pressure cell before use in ELISA (1). The amount of methane in the headspace of cultures was determined by gas chromatography (8).

RESULTS

Kinetics of methanogenesis during phosphate limitation. The rates of methanogenesis by fed-batch cultures of *M.*

barkeri are presented throughout this paper to emphasize the transition from an exponentially increasing rate of methane production to a constant rate which occurs under phosphate-limiting conditions. The data are first plotted as the accumulation of CH_4 with time, and gradients of that curve are then taken at different times throughout the fed-batch cycle. The kinetics of methanogenesis split into three periods: an exponentially-increasing rate, a constant (maximum) rate, and a declining rate. The declining period is due to depletion of the growth substrate ($\text{H}_2\text{-CO}_2$).

The transition in kinetics from exponentially increasing to a constant rate of methanogenesis is not due to substrate limitation. To examine whether the concentration of phosphate in the medium affects the transition, I compared the kinetics of methanogenesis from cultures containing various initial amounts of phosphate (Fig. 1). Concentrations of phosphate from 0 to 50 μM were examined. Increasing the concentration of phosphate from 0 to 10 μM in 4.5-ml cultures increased the maximum rate of methanogenesis achieved and shortened the period of constant methane production rate from over 6 days to less than 1 day. With 10 μM phosphate in the 4.5-ml cultures the initial C (in CO_2)/P ratio was 1,600.

Addition of phosphate to a culture (400 ml) in the constant period of methanogenesis changed the kinetics (Fig. 2). The addition of phosphate initiated an exponentially increasing period of methanogenesis with a consequent increase in the maximum rate achieved and a shortening of the total time taken to end methane production because of substrate starvation.

Uptake of ^{32}P . The uptake of ^{32}P (added to the medium as $\text{KH}_2^{32}\text{PO}_4$) was measured in 400-ml cultures. Removal of

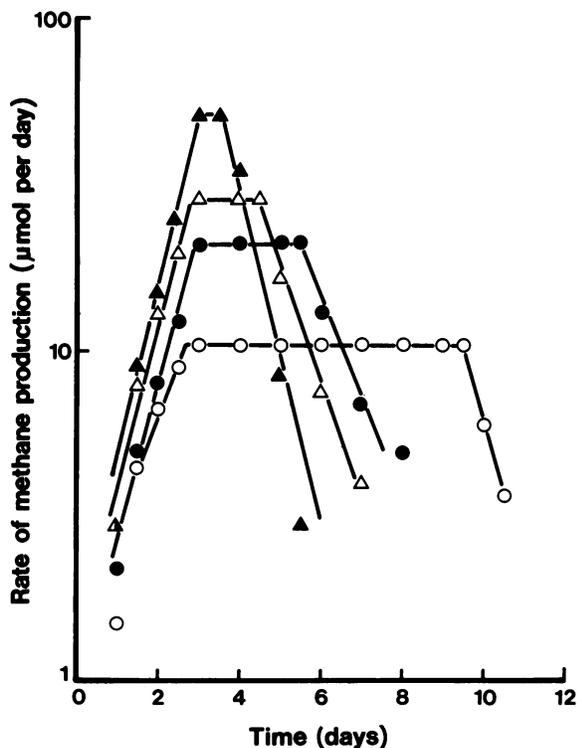


FIG. 1. Rate of methanogenesis from 4.5-ml cultures of *M. barkeri* containing 0 μM (○), 0.5 μM (●), 5 μM (△), and 10 μM (▲) initial concentrations of phosphate.

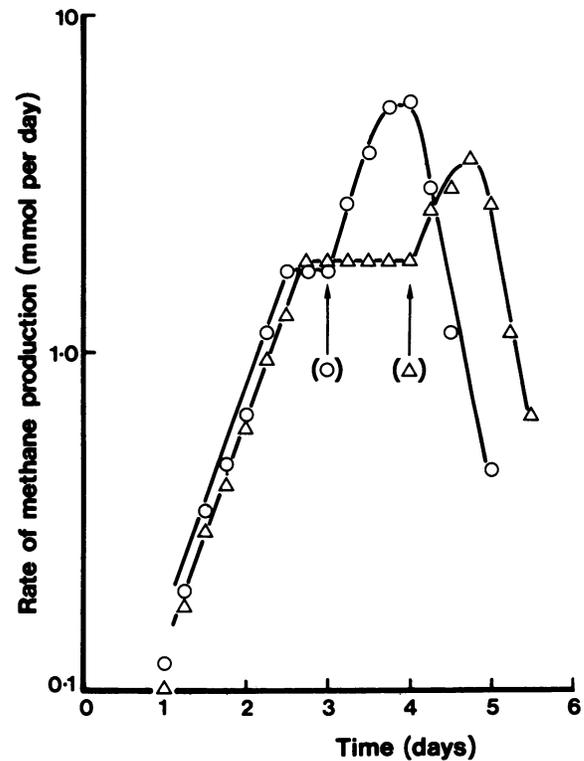


FIG. 2. Rate of methanogenesis from two 400-ml cultures of *M. barkeri* without initial phosphate. Phosphate (to 10 μM) was added at the times indicated by the arrows.

radiolabel from the medium and incorporation into cell pellets was measured. In addition, the rate of methanogenesis and accumulation of cell protein, determined by ELISA, were measured. The results at two different initial phosphate concentrations are given in Fig. 3 and 4. With an initial concentration of 1.25 μM phosphate the transition from an exponentially increasing to a constant rate of methanogenesis coincided with the removal of phosphate from the medium and its appearance in cells. The rate of uptake of radiolabel from the medium increased more rapidly than the rate of methanogenesis. From the onset of the constant period of methane production radiolabel was released from cells to the medium with a rate constant of approximately 0.2 day^{-1} . The accumulated cell concentration remained constant at about $13 \mu\text{g}$ of protein ml^{-1} from the onset of the constant period of methanogenesis. No figure for cell protein was obtained at the first time point (0.7 day). This is because samples were diluted for passage through a French pressure cell, and the cell protein concentration was then estimated to be below the minimum detection limit in ELISA (1).

There was no extended period of constant rate of methanogenesis at the higher (11.25 μM) initial phosphate concentration (Fig. 4). The rate of uptake of radiolabel from the medium increased more rapidly than the rate of methanogenesis. During the period of decline in the rate of methanogenesis, radiolabel was released into the medium with a rate constant of 0.8 day^{-1} . The concentration of cell protein continued to accumulate throughout the fed-batch cycle and proceeded linearly from day 2, the time at which the period of exponentially increasing rate of methanogenesis ended.

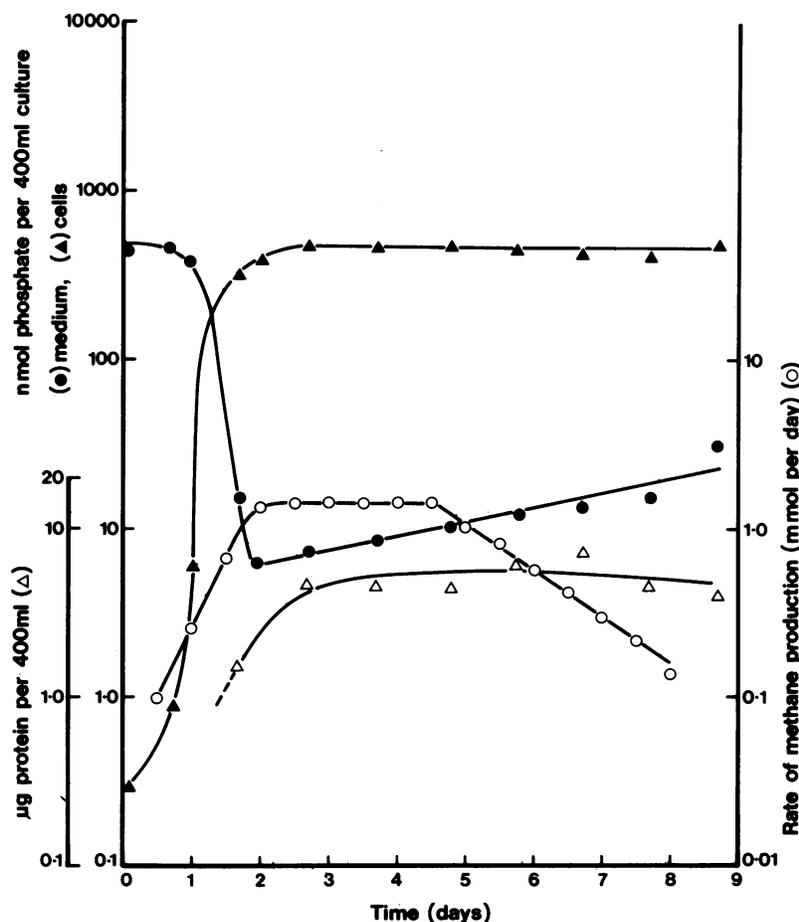


FIG. 3. Incorporation of ^{32}P as $\text{KH}_2^{32}\text{PO}_4$ from medium supernatant (●) to *M. barkeri* (▲) in a 400-ml culture initially containing $1.25 \mu\text{M}$ phosphate. Rate of methanogenesis (○) and cell concentration (Δ) are also shown.

DISCUSSION

Anaerobic digestion is used to treat many different wastes to reduce pollution and to produce methane as a fuel. Two problems associated with anaerobic digestion are the length of time taken to start up a digester and the production of excess biomass, which itself constitutes a waste, by fully commissioned digesters. Coupling of methanogenesis to cell growth can have a profound effect on both aspects of anaerobic digestion; ideally, methanogenesis should be tightly coupled to cell growth during start-up and uncoupled in fully operational digesters. The treatment of naturally phosphate-deficient wastes such as landfill leachates (3, 11), some starch wastes (9), and some molasses-based effluents (12, 20) may be suited to control of coupling by addition, or omission, of supplementary phosphate. Phosphate limitation may affect rates of anaerobic degradation in natural ecosystems, although other factors may also be important there and in digesters. The effect of phosphate limitation on a pure culture of *M. barkeri* is described here.

The effects of a range of initial phosphate concentrations were examined in fed-batch cultures. The period of constant rate of methanogenesis shortened when the phosphate in the medium was increased from 0.1 to $10 \mu\text{M}$, and the maximum rate of methanogenesis attained increased but not in proportion to the amount of phosphate in the medium. *M. barkeri*

contains approximately 40% (wt/wt) carbon and 1.2 to 1.9% (wt/wt) phosphorus (13). From published values of Y (grams of cells per mole of CH_4) for *M. barkeri* grown on $\text{H}_2\text{-CO}_2$ (4, 21) it can be calculated that up to 22% of the carbon utilized in a medium will be for cell synthesis. The required ratio of C/P in a medium is then 100 to 150. C/P ratios an order of magnitude higher were found experimentally in this work to be required for extended periods of constant-rate methanogenesis. In phosphate-limited growth of *M. thermoautotrophicum* in a chemostat, the K_s (phosphate) was estimated to be $4 \mu\text{M}$, and the cell density was proportional to the incoming phosphate concentration between 25 and $100 \mu\text{M}$ (17). In the fed-batch studies, a transition from phosphate-sufficient to phosphate-deficient conditions is being followed, and results need not be comparable with those with steady states in a chemostat. Addition of phosphate to cultures in the constant period of methanogenesis resulted in a transition to an exponentially increasing rate. This suggests that the constant period is due to phosphate limitation and that the phosphate limitation is reversible; comparable results were obtained with the use of energy-yielding substrates by eubacteria (5, 18).

The uptake of ^{32}P (in $\text{KH}_2^{32}\text{PO}_4$) was followed and confirmed the supposition that the transition in kinetics of methanogenesis coincided with phosphate depletion in the medium. The kinetics of uptake of radiolabel were not

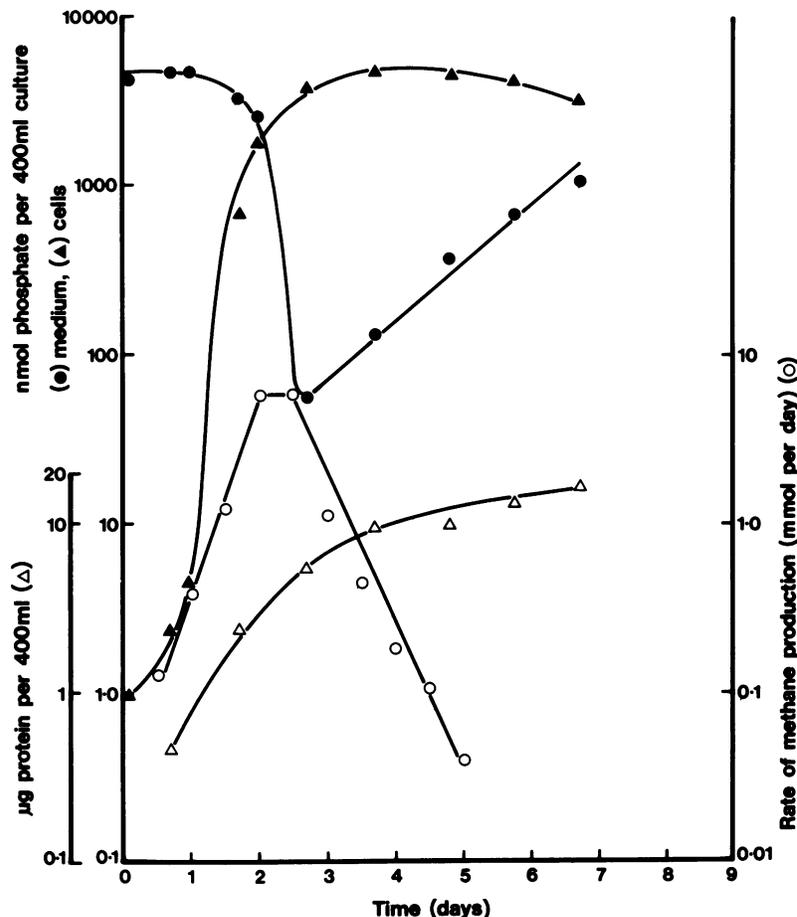


FIG. 4. Incorporation of ^{32}P as $\text{KH}_2^{32}\text{PO}_4$ from medium supernatant (●) to *M. barkeri* (▲) in a 400-ml culture initially containing $11.25 \mu\text{M}$ phosphate. Rate of methanogenesis (○) and cell concentration (Δ) are also shown.

coincident with the exponentially increasing rates of methanogenesis. The radiolabel uptake rates increased more rapidly than the rates of methanogenesis. This is possibly due to the accumulation of a phosphate storage compound such as cyclic-2,3-diphosphoglycerate. Radiolabel was released from cells to the medium after completion of the exponential period of methanogenesis, and the rate of release was higher in the culture initially containing the higher concentration of phosphate. This may have been due to autolysis of cells when not growing; this is known to occur with some strains of *M. barkeri* (2, 15, 19).

A constant rate of methanogenesis may have several plausible explanations, but perhaps the simplest is that although methanogenesis is occurring cell growth is not. At a low ($1.25 \mu\text{M}$) initial concentration of phosphate in the medium the cell protein concentration did not increase during the constant period of methanogenesis, which indicates uncoupling of methanogenesis from cell growth. During this period the specific rate of methane production was $0.27 \text{ mol of CH}_4 \text{ per g of protein per day}$ which is within the range of values published for other strains of *M. barkeri* (summarized elsewhere [1]). Methanogenesis was therefore not severely impaired by phosphate starvation. At a higher ($11.25 \mu\text{M}$) initial concentration of phosphate the concentration of cell protein continued to rise after the end of the exponential period of methanogenesis at a constant rate and is comparable to the observation of linear growth of *M.*

thermoautotrophicum during phosphate starvation (17); in that case reserves of cyclic-2,3-diphosphoglycerate within the cells were utilized.

ACKNOWLEDGMENTS

I am grateful to Godfrey Powell for discussions throughout the work and to Helen Jones for technical assistance.

LITERATURE CITED

1. Archer, D. B. 1984. Detection and quantitation of methanogens by enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* **48**:797-801.
2. Archer, D. B., and N. R. King. 1984. Isolation of gas vesicles from *Methanosarcina barkeri*. *J. Gen. Microbiol.* **130**:167-172.
3. Bull, P. S., J. V. Evans, R. M. Wechsler, and K. J. Cleland. 1983. Biological technology of the treatment of leachate from sanitary landfills. *Water Res.* **17**:1473-1481.
4. Daniels, L., R. Sparling, and G. D. Sprott. 1984. The bioenergetics of methanogenesis. *Biochim. Biophys. Acta* **768**:113-163.
5. Forrest, W. W. 1969. Energetic aspects of microbial growth, p. 65-86. In P. M. Meadow and S. J. Pirt (ed.), *Microbial growth*. Society for General Microbiology Symposium 19. Cambridge University Press, Cambridge.
6. Havilland, R. T., and L. L. Bieber. 1970. Scintillation counting of ^{32}P without added scintillator in aqueous solutions and organic solvents and on dry chromatographic media. *Anal. Biochem.* **33**:323-334.
7. Kell, D. B., H. J. Doddema, J. G. Morris, and G. D. Vogels.

1981. Energy coupling in methanogens, p. 159–170. In H. Dalton (ed.), *Microbial growth on C₁ compounds*. Heyden and Son Ltd., London.
8. Kirsop, B. H., M. G. Hilton, G. E. Powell, and D. B. Archer. 1984. Methanogenesis in the anaerobic treatment of food-processing wastes. *Soc. Appl. Bacteriol. Tech. Ser.* **19**:139–158.
 9. Lettinga, G., L. W. Hulshoff Pol, W. Wiegant, W. de Zeeuw, S. W. Hobma, P. Grin, R. Roersma, S. Sayed, and A. F. W. van Velsen. 1983. Upflow sludge blanket processes, p. 139–158. In *Proceedings of the 3rd International Symposium on Anaerobic Digestion*, Boston.
 10. Powell, G. E., M. G. Hilton, D. B. Archer, and B. H. Kirsop. 1983. Kinetics of the methanogenic fermentation of acetate. *J. Chem. Tech. Biotechnol.* **33B**:209–215.
 11. Robinson, H. D., C. Barber, and P. J. Maris. 1982. Generation and treatment of leachate from domestic wastes in landfills. *Water Pollut. Control* **81**:465–478.
 12. Sanchez Riera, F., S. Valz-Gianinet, D. Callieri, and F. Sineriz. 1982. Use of a packed-bed reactor for anaerobic treatment of stillage of sugar cane molasses. *Biotechnol. Lett.* **4**:127–132.
 13. Scherer, P., H. Lippert, and G. Wolff. 1983. Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductively coupled plasma emission spectrometry. *Biol. Trace Elem. Res.* **5**:149–163.
 14. Scherer, P., and H. Sahm. 1981. Effect of trace elements and vitamins on the growth of *Methanosarcina barkeri*. *Acta Biotechnol.* **1**:57–65.
 15. Scherer, P., and H. Sahm. 1981. Influence of sulphur-containing compounds on the growth of *Methanosarcina barkeri* in a defined medium. *Eur. J. Appl. Microbiol. Biotechnol.* **12**:28–35.
 16. Schönheit, P., J. Moll, and R. K. Thauer. 1980. Growth parameters (K_s , μ_{max} , Y_s) of *Methanobacterium thermoautotrophicum*. *Arch. Microbiol.* **127**:59–65.
 17. Seely, R. J., and D. E. Fahrney. 1984. Levels of cyclic-2,3-diphosphoglycerate in *Methanobacterium thermoautotrophicum* during phosphate limitation. *J. Bacteriol.* **160**:50–54.
 18. Senez, J. C., and J.-P. Belaich. 1963. Étude microcalorimétrique de la croissance bactérienne et du contrôle de l'activité métabolique par le phosphate. *Colloq. Int. C.N.R.S.* **124**:357–369.
 19. Smith, M. R., and R. A. Mah. 1978. Growth and methanogenesis by *Methanosarcina* strain 227 on acetate and methanol. *Appl. Environ. Microbiol.* **36**:870–879.
 20. Szendrey, L. M. 1983. Startup and operation of the Bacardi Corporation anaerobic filter, p. 365–377. In *Proceedings of the 3rd International Symposium on Anaerobic Digestion*, Boston.
 21. Taylor, G. T. 1982. The methanogenic bacteria. *Prog. Ind. Microbiol.* **16**:231–239.