Factors Influencing Filament Length of *Methanospirillum hungatii*

By G. B. PATEL, L. A. ROTH* AND G. D. SPROTT

*Division of Biological Sciences, National Research Council of Canada,
Ottawa, Ontario K1A 0R6, Canada*

(Received 24 January 1979)

Cultivation of *Methanospirillum hungatii* GP1 at 35 °C in low PO_4^{3-} (3.0 mM) medium resulted in growth as motile curved rods (6 to $8 \times 0.5 \mu\text{m}$). However, predominantly long filaments averaging 70 to 300 μm were observed in the presence of high concentrations of PO_4^{3-} (43.7 mM) or Na^+ (225 mM) or after incubation at 25 °C in low PO_4^{3-} medium. These conditions also resulted in decreased growth rates. Tests with cell-free extracts of long and short form filaments indicated that long filament formation is probably related to a deficiency in heat-labile factor(s) required for cell separation.

INTRODUCTION

The cell morphology of many micro-organisms can be manipulated by the control of growth conditions such as nutrient supply (Webb, 1951) and incubation temperature (Gounot, 1976). Some of the morphological changes have been attributed to failure of septation (Ferroni & Inniss, 1974) and to decreased activity of cell separation enzymes (Lominski *et al.*, 1958). Morphological changes have been studied only superficially in the obligately anaerobic, methanogenic bacteria. Filament formation and coiling occur in *Methanobacterium thermoautotrophicum* (Zeikus & Wolfe, 1973); chaining in *Methanobacterium formicicum* (Mylroie & Hungate, 1954), *Methanobacterium ruminantium* (Smith & Hungate, 1958), *Methanobacterium arbophilicum* (Zeikus & Henning, 1975); and filament formation in *Methanospirillum hungatii* JF1 (Ferry *et al.*, 1974). In most cases the above morphological types were neither predominant nor selectively enhanced over other types present, with the exceptions of temperature-controlled morphology changes in *M. thermoautotrophicum* (Zeikus & Wolfe, 1973) and nutrient-mediated morphology changes of *Methanobacterium* strain AZ (Zehnder & Wuhrmann, 1977). This paper reports on the selective cultivation of *M. hungatii* as predominantly long filaments and on some of the factors responsible for this morphological change.

METHODS

Media. The composition of sodium carbonate-buffered synthetic acetate broth (SA, 3.0 mM- PO_4^{3-}) used for culture maintenance was described by Patel & Roth (1977). High phosphate broth (PA, 43.7 mM- PO_4^{3-}) was of the same composition as SA, except that the phosphate level was increased by using $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (0.05 M, pH 7.0) to dissolve the ingredients. All media were pre-reduced and, unless stated otherwise, 10 ml samples were dispensed under H_2/CO_2 (4:1, v/v) into 160 ml serum vials fitted with butyl rubber stoppers. The pH values of the media after autoclaving (103 kPa for 15 min) were 6.8 to 7.0 at 25 °C.

Culture maintenance and inocula preparation. Stock cultures of *Methanospirillum hungatii* GP1 (Patel *et al.*, 1976; National Research Council of Canada Culture Collection no. 2214) were maintained by weekly transfers into SA broth. Cultures for inoculum or cell extract preparation were cultivated for 1 week at 35 °C under H_2/CO_2 (4:1, v/v) in SA broth if short filaments were desired and in PA broth if long filaments were needed. In most instances the inoculum was 1 to 2% (v/v) to give an initial count of about 5×10^8 filaments ml^{-1} in the test medium.

* Present address: Alberta Agriculture, Food Laboratory, O.S. Longman Building, Edmonton, Alberta T6H 4P2, Canada.

Fig. 1. *Methanospirillum hungatii* GP1 filaments stained with Victoria blue to visualize the cell spacers: (a) grown in SA broth; (b) grown in PA broth; (c) grown in PA broth but treated with 0.75 M-sucrose before staining.

Experimental analyses. Growth was estimated turbidimetrically (A_{660}); these estimates correlated well with cell protein determinations (Lowry *et al.*, 1951). Methane in the culture head-space was assayed as described by Patel & Roth (1977). Cell spacers in the filaments (equivalent to septa in Gram-positive bacteria) were visualized by bright-field microscopy, after fixing washed filaments (Tris/HCl buffer, 0.01 M, pH 8.0) with Bouin's fluid by diffusion through agar blocks and staining (10 to 15 s) with 0.05% Victoria blue (Robinow & Murray, 1953). In some experiments the cells were plasmolysed by incubation in 0.75 M-sucrose for 30 min prior to fixation.

Measurement of filament length. Filament length was measured with an ocular micrometer using phase-contrast optics. However, this method was not suitable for the accurate measurement of filaments in excess of 100 μm or for those not lying in a straight path. This problem was overcome by measuring filaments ranging in length from 8 to 500 μm by careful alignment with the ocular micrometer and also counting the number of wave crests and troughs in each filament. (The latter is easily accomplished using phase contrast optics, since most of the filaments appear in a regular wave-like arrangement.) The average distance between the highest point of a crest and the lowest point of an adjacent trough was found to be $8.75 \pm 0.13 \mu\text{m}$ (968 measurements in 90 filaments). Consequently, the number of crests and troughs in a filament were counted and multiplied by 8.75 to estimate filament length.

Cell extract preparation. Filaments were harvested by centrifuging (23 000 g , 30 min), washed once in Tris/HCl buffer and disrupted ultrasonically for 3 min in 30 s bursts (95% breakage). The extracts were stored under H_2/CO_2 (4:1, v/v) and used the same day.

Electron microscopy. Thin sections of *M. hungatii* were prepared and examined as described by Colvin & Leppard (1977).

RESULTS AND DISCUSSION

Methanospirillum hungatii GP1 grew in SA broth as single motile rods (6 to $8 \times 0.5 \mu\text{m}$) and filaments (16 to $18 \times 0.5 \mu\text{m}$) (Fig. 1a). Occasional filaments up to 100 μm were also seen. Electron microscopic examination of ultra-thin sections indicated that each filament was composed of individual cells enclosed in a sheath-like structure and separated by discrete, unique partitions, called cell spacers. In these aspects the ultrastructure of *M. hungatii* strain GP1 is similar to that of strain 3P3 described by Zeikus & Bowen (1975). The cell spacers are not usually evident by phase-contrast or bright-field microscopy without special staining procedures.

In contrast to growth as short filaments in SA broth, *M. hungatii* grew primarily as long filaments (at least 100 μm) when cultured in PA broth. Victoria blue staining of the long filaments indicated that the cell spacers were about 9 μm apart, as observed for the short filaments grown in SA broth (Fig. 1). The ion which induced the growth of long filaments

Table 1. Mean filament lengths of *M. hungatii* GP1 in different media

Supplements [short-filament extract (SFE), long-filament extract (LFE) or 'supernatant'] were added to the growth medium (10 ml in 160 ml serum vials) before inoculation with short filaments grown in SA broth. Heated extracts were maintained at 85 °C for 5 min. 'Supernatant' was 0.2 ml of SA growth medium from which short filaments had been harvested. H₂ and CO₂ were replenished daily. Filaments were measured after 7 d incubation at 35 °C; the results show the mean length $\pm$ standard error, and (in parentheses) the number of filaments counted.

Growth medium	Supplement [μg protein (ml broth) ⁻¹]	Filament length (μm)	Methane accumulated (mmol)
SA	None	16.10 $\pm$ 0.80 (60)	1.40
PA	None	218 $\pm$ 15 (60)	1.41
PA	SFE (50)	177 $\pm$ 22 (20)	1.13
PA	SFE (200)	88.6 $\pm$ 2.1 (80)	1.07
PA	Heated SFE (200)	150.0 $\pm$ 9.1 (60)	1.14
PA	LFE (180)	222 $\pm$ 19 (40)	1.33
PA	Heated LFE (180)	214 $\pm$ 22 (40)	1.00
PA	'Supernatant'	220 $\pm$ 29 (20)	1.00

was PO₄³⁻ rather than K⁺, since growth in SA broth supplemented with up to 100 mM-KCl yielded predominantly short filaments. Electron microscopic examination of ultra-thin sections indicated no obvious structural differences between the cell walls of long and short filaments.

Experiments were conducted to determine if filament length correlated with the growth rate of the culture. Biomass doubling times, determined turbidimetrically in 250 ml flasks (100 ml medium) fitted with side-arms, were 16, 32 and 136 h in SA broth, PA broth and SA broth containing 225 mM-Na⁺, respectively. This confirmed a previous observation by Patel & Roth (1977) that high NaCl concentrations inhibited growth and methane production of *M. hungatii* GP1. The final amount of growth attained after 7 d incubation was comparable in SA and PA broth, but in the presence of 225 mM-NaCl the final growth was decreased to one-third. Filament lengths (see Table 1 for SA and PA broth) increased significantly as the growth rate decreased. In the medium containing 225 mM-NaCl, the mean filament length was 277 μm (not shown).

The possibility that high PO₄³⁻ and high NaCl concentrations directly affected cell separation events was excluded by measuring filament lengths and growth rates of *M. hungatii* after cultivation in SA broth at 35 °C and 25 °C. At the sub-optimal temperature of 25 °C the biomass doubling time was 45 h, measured in the exponential phase after a 260 h lag period, compared with about 20 h at 35 °C. This decrease in growth temperature also led to an increase in the mean filament length from 16 μm at 35 °C to 82 $\pm$ 10 μm at 25 °C.

Long filaments (from PA broth) re-inoculated into SA broth [H₂/CO₂ (4:1, v/v)] grew as short filaments. However, resuspension of long filaments in SA broth under conditions that did not support growth [N₂/CO₂ (4:1, v/v)] did not lead to the formation of short filaments. The effect of high PO₄³⁻ concentrations on filament length is therefore not due to an inhibition in the activity of cell separation factors.

Since the cell spacers were about one cell length apart (Fig. 1), growth as long filaments was probably not caused by a failure in cross-wall formation, as reported for some rod-shaped bacteria (Ferroni & Inniss, 1974; Gounot, 1976). To investigate whether the reduced growth rates might correlate with a decreased synthesis of factor(s) responsible for cell separation, cells were grown in PA broth in the presence of extracts prepared from both short and long filaments (Table 1). The mean filament lengths of cells grown in PA broth (control) or in PA broth in the presence of a short-filament extract were significantly different ($P < 0.01$). Further, the filament lengths were related to the amount of extract added. A similar significance ($P < 0.01$) was noted between the means of samples which received heated and unheated short-filament extract (200 μg protein ml⁻¹). In contrast, growth in

the presence of extract prepared from long filaments, or from culture supernatant, resulted in mean filament lengths that did not differ significantly from the control culture (PA broth). Since a decrease in length occurred in the presence of 44 mM- PO_4^{3-} , this provides further evidence that growth as long filaments in PA broth is not due to PO_4^{3-} acting as an inhibitor of cell separation factors. It seems probable that at decreased growth rates the synthesis of a heat-labile component present in short-filament extracts is also decreased.

From these results, the possibility cannot be ruled out that the active extract causes an increased susceptibility of the cell wall to enzymic attack. Treatment with lysozyme, however, did not result in unchaining or cell lysis in long or short filaments of *M. hungatii*, even after pretreatment with β -glucuronidase from *Helix pomatia*, protease, trypsin, phospholipase A and C, or with EDTA. Further, penicillin G at 1000 $\mu\text{g ml}^{-1}$ had little or no effect on cell growth and methane formation. These findings corroborate the recent report by Kandler & König (1978) that the outer wall (or sheath) of *M. hungatii* lacks peptidoglycan.

Preliminary tests with another strain of *M. hungatii* (type strain JF1) indicated that it, too, can be selectively induced to grow as long filaments by incubation in high PO_4^{3-} medium. High PO_4^{3-} and Na^+ concentrations that caused filament formation in *M. hungatii* GPI had no noticeable effect on the morphologies of three other methanogenic cultures tested; namely, *Methanobacterium thermoautotrophicum*, *Methanobacterium* strain M.O.H. and a strain similar to *Methanobacterium formicicum*.

CONCLUSIONS

Growth as long filaments is expected to have ecological significance. Filamentation should decrease the dispersal of the organism into the surrounding environment, both by decreasing the number of cell filaments and decreasing motility. The results also have significance in taxonomic and isolation work where predominant morphological types observed in natural ecosystems are seldom seen during laboratory cultivation in synthetic media. In part, this may be attributed to variations between the growth rates of the micro-organisms in their natural environment and those in laboratory media.

The authors appreciate the assistance of Dr J. R. Colvin and Mr L. C. Sowden with electron microscopy, and Mr R. H. Whitehead for taking photomicrographs. Technical assistance of Mr B. Agnew and Kathleen Shaw is also acknowledged. This paper is N.R.C.C. no. 17369.

REFERENCES

- COLVIN, J. R. & LEPPARD, G. G. (1977). The biosynthesis of cellulose by *Acetobacter xylinum* and *Acetobacter acetogens*. *Canadian Journal of Microbiology* **23**, 701–709.
- FERRONI, G. D. & INNISS, W. E. (1974). Reversal of filament formation in the psychrophile *Bacillus insolitus*. *Canadian Journal of Microbiology* **20**, 1281–1283.
- FERRY, J. G., SMITH, P. H. & WOLFE, R. S. (1974). *Methanospirillum*, a new genus of methanogenic bacteria, and characterization of *Methanospirillum hungatii* sp. nov. *International Journal of Systematic Bacteriology* **24**, 465–469.
- GOUNOT, A. M. (1976). Effects of temperature on the growth of psychrophilic bacteria from glaciers. *Canadian Journal of Microbiology* **22**, 839–846.
- KANDLER, O. & KÖNIG, H. (1978). Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. *Archives of Microbiology* **118**, 141–152.
- LOMINSKI, I., CAMERON, J. & WYLLIE, G. (1958). Chaining and unchaining *Streptococcus faecalis* – a hypothesis of the mechanism of bacterial cell separation. *Nature, London* **181**, 1477.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MYLROIE, R. L. & HUNGATE, R. E. (1954). Experiments on the methane bacteria in sludge. *Canadian Journal of Microbiology* **1**, 55–64.
- PATEL, G. B. & ROTH, L. A. (1977). Effect of sodium chloride on growth and methane production of methanogens. *Canadian Journal of Microbiology* **23**, 893–897.
- PATEL, G. B., ROTH, L. A., VAN DEN BERG, L. & CLARK, D. S. (1976). Characterization of a strain of *Methanospirillum hungatii*. *Canadian Journal of Microbiology* **22**, 1404–1410.
- ROBINOW, C. F. & MURRAY, R. G. E. (1953). The

- differentiation of cell wall, cytoplasmic membrane and cytoplasm of Gram-positive bacteria by selective staining. *Experimental Cell Research* **4**, 390–407.
- SMITH, P. H. & HUNGATE, R. E. (1958). Isolation and characterization of *Methanobacterium ruminantium* n.sp. *Journal of Bacteriology* **75**, 713–718.
- WEBB, M. (1951). The influence of magnesium on cell division. 6. The action of certain hydrolytic enzymes on the filamentous and chain forms of Gram-positive rod-shaped organisms. *Journal of General Microbiology* **5**, 496–501.
- ZEHNDER, A. J. B. & WUHRMANN, K. (1977). Physiology of a *Methanobacterium* strain AZ. *Archives of Microbiology* **111**, 199–205.
- ZEIKUS, J. G. & BOWEN, V. G. (1975). Fine structure of *Methanospirillum hungatii*. *Journal of Bacteriology* **121**, 373–380.
- ZEIKUS, J. G. & HENNING, D. L. (1975). *Methanobacterium arbophilicum* sp.nov. An obligate anaerobe isolated from wetwood of living trees. *Antonie van Leeuwenhoek* **41**, 543–552.
- ZEIKUS, J. G. & WOLFE, R. S. (1973). Fine structure of *Methanobacterium thermoautotrophicum*: effect of growth temperature on morphology and ultrastructure. *Journal of Bacteriology* **113**, 461–467.