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Influence of Sulphur-Containing Compounds on the Growth of *Methanosarcina barkeri* in a Defined Medium

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Summary. Optimal growth of *Methanosarcina barkeri* occurred in a defined medium containing methanol when 2.5–4 m*M* sodium sulphide was added giving a concentration of 0.04–0.06 m*M* dissolved sulphide ($HS^- + S^{2-}$. When the sulphide concentration was too low for optimal growth (e.g., 0.1 m*M* Na_2S added) the addition of the redox resin 'Serdoxit' acted as a sulphide reservoir and caused a significant stimulation of growth. Furthermore it could be demonstrated that iron sulphide, zinc sulphide or L-methionine could also act as sulphur sources while the addition of sodium sulphate to sulphide-depleted media failed to restore growth. The amino acid L-cysteine (0.85 m*M*) stimulated growth but could not replace Na_2S.

Under optimal cysteine- and sulphide concentrations the generation time of this strain was about 7–9 h during growth on methanol, giving a growth yield of about 0.14 g/g methanol consumed. Different *M. barkeri* strains were also able to grow under these conditions on acetate (30–50 h doubling time) without a significant lag-phase and with complete substrate consumption even though the inoculum was grown on methanol or H_2–CO_2. When methanol and acetate were present as a mixture in the medium both were used simultaneously.

Introduction

Anaerobic digestion of organic matter has been practised for many years as a process for sewage treatment and has found its main application in the stabilization of sewage sludge. In recent years energy considerations and environmental concerns have increased interest in the direct anaerobic treatment of industrial waste water carrying high concentrations of dissolved organic matter. However, although methane fermentation has been used for a long time the fundamental biochemistry and microbiology and the role played by the various elements in the process are still poorly understood. For example the literature on anaerobic fermentation indicates conflictingly both the necessity, as well as the undesirability, of the presence of sulphate and sulphide for the degradation of organic compounds to methane (Mah et al. 1977; Zeikus 1977; Zehnder 1978). In the cultivation of methanogenic bacteria (Barker 1940; Schnellen 1947) sulphide has been implicated in the synthesis of sulphur containing organic compounds such as coenzyme M, which is involved in its methylated form as the terminal electron acceptor during methanogenesis (McBride and Wolfe 1971; Balch and Wolfe 1979). Furthermore sulphur containing compounds are generally used to maintain the reducing environment essential for growth (Mah et al. 1977; Zeikus 1977; Zehnder 1978).

On the other hand it has been observed that sulfate inhibits methanogenesis in an anaerobic microbial ecosystem and that sulphide is inhibitory at fairly low concentration (0.1 m*M* HS^-) to an isolated *Methanobacterium* growing on acetate (Cappenberg 1975). Recently Mountfort and Asher (1979), reported that the methanogenic bacterium *Methanosarcina barkeri* has a specific physiological requirement for sulphide in a complex medium. However, an increase of the added sulphide to 12.5 m*M* led to a decrease of the specific growth rate.

This multiple role of inorganic sulphur necessitated work to determine the influence of various sulphur containing compounds on methanogenic bacteria. Acetic acid has been shown to be the precursor of most (up to 70%) of the methane produced in anaerobic sludge digesters (Mah et al. 1977; Zeikus 1977; Zehnder 1978; Smith et al. 1980), and the present work explored the effects of a variety of sulphur containing compounds on the growth

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and methane production of *M. barkeri*, one of the few methanogenic bacteria for which methane production and growth on acetate is described.

Materials and Methods

Organisms. Cultures of *Methanosarcina barkeri* DSM 804, DSM 800, DSM 805, and DSM 1232 were obtained from the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG).

Culture Medium and Growth Conditions. A modified Hungate technique in combination with the serum bottle technique (Miller and Wolin 1974) was used for medium preparation and cultivation under strictly anaerobic conditions.

The culture medium (100 ml) was precooled to 4 °C and then degassed for 5 min with the help of a water jet pump and the highest speed of a magnetic stirrer. The medium consisted of the following components dissolved in deionized and quartz distilled water namely: Imidazole-HCl buffer, 40 m*M*; NaH_2PO_4, 0.5 m*M*; Na_2HPO_4, 0.5 m*M*; $MgCl_2$, 1.7 m*M*; $CaCl_2$, 2.0 m*M*; KCl, 5.4 m*M*; NaCl, 34 m*M*; NH_4Cl, 9.4 m*M*; together with trace elements: H_3BO_3, 1.45 μM; $CoCl_2$, 0.25 μM; Na_2SeO_3, 0.1 μM; $ZnCl_2$, 0.1 μM; $MnCl_2$, 0.045 μM; $NaMoO_4$, 0.037 μM; $NiCl_2$, 0.025 μM; $CuCl_2$, 0.017 μM; vitamin solution, 10 ml/l (Wolin et al. 1963); resazurin, 4.4 μM; titanium(III)-citrate, 0.15 m*M* (modified, Zehnder and Wuhrmann 1976); L-cysteine-HCl, 0.85 m*M*; $(NH_4)_2Fe(SO_4)_2 \times 6H_2O$, 0.1 m*M*; $Na_2S \times 9H_2O$, 2.5 m*M*; and as a carbon and energy source methanol, 180 m*M*, sodium acetate, 45 m*M*, or H_2–CO_2 (80 : 20). The concentrations of the following medium components were checked as optimal for growth: Imidazole-HCl, $CaCl_2$, $MgCl_2$ KCl, Na-phosphate, Ti-citrate, L-cysteine and ferrous ammonium sulphate.

The bottle containing the medium was filled with argon and then the redox solutions Ti(III)-citrate (0.1 M), L-cysteine (0.4 M) and ammonium ferrous sulphate (0.1 M) were added. The redox potential of the culture medium was at this stape −400 to −500 mV, related to an AgCl electrode. Test tubes with a serum bottle neck (19 ml, 12 mm i.d.) were filled under argon with 5 ml medium and closed by commonly used rubber stoppers (1 = 20 mm.) The methanol in the test tubes was reduced by approximately 10% of the initial amount during the degassing of the medium. The substances tested for their influence on growth (e.g., Na_2S) were injected as solutions with high concentrations (e.g., 0.25 M), before sterilization (15 min at 120 °C). Possible pH changes were corrected before autoclaving with oxygen free 1 N HCl or 1 N NaOH.

Cultures of *M. barkeri* were inoculated by syringe (needle ϕ = 0.6 mm) with 5% v/v of inoculum in the late log phase or early stationary phase of growth. After inoculation the low excess pressure in the test tubes was released by inserting a needle. During growth (generally at 30 °C) the pressure in the closed culture vessels increased to approximately 0.9–1.2 atm overpressure.

Analytical Procedures. The gas production was measured manometrically using a pressure gauge (L'Air Liquide, Mülheim, FRG) connected by a Luer Lock adapter with a Luer Lock canule which had to be heated slightly to stick through the rubber stopper. The measured values of gas volume were corrected by the inner total volume of the manometer (e.g., 1.05 ml at 1 atm overpressure).

Methanol, methane, carbon dioxide and acetate were measured by gas chromatogrphy using a Hewlett Packard model 5840A gas chromatograph. Methanol and methane were determined on a 2.5 m × 2 mm glass column, packed with Porapak QS (100/120 mesh), using a flame ionization detector. The injector temperature was 300 °C, the oven temperature was 160 °C and the detector temperature was 300 °C. Nitrogen was used as carrier gas at a flow rate of 48 ml/min. Acetate was analysed on the same column. The samples were injected at 300 °C and the temperatures of the oven and the flame ionization detector were 210 °C and 300 °C respectively. The samples contained in 1 N HCl 1% propionic acid as the internal standard. Acetate was also measured enzymatically with acetate kinase as described by Miller and Wolin (1980). Carbon dioxide was determined on a 2.6 × 2 mm column, packed with silica gel (80/100 mesh; 150–200 μm), using a thermal conductivity detector. The injector temperature was 200 °C, the oven temperature was 130 °C and the detector temperature was 110 °C. Helium was used as carrier gas for the CO_2 determinations at a flow rate of 5–7 ml/min.

The methionine content in the culture medium was analyzed using an amino acid analyser (model BC 200 of Biocall).

The total amount of dissolved sulphide ($HS^- + S^{2-}$) was determined in solutions containing 2.5 N NaOH, 0.1 M EDTA and 0.2 M ascorbic acid (Bock and Puff 1968) using a sulphide sensitive electrode (Orion, Cambridge, Mass., USA). Additionally the methylene blue method was used (Koch and Koch-Dedic 1974).

Determinations of cell yields were carried out by filtering 2–5 ml culture samples during the stationary phase of growth with preweighed cellulose nitrate filters (1.2 μM, Satorius, Göttingen, FRG). The filters were washed with 5–10 ml distilled water (no lysis occurred during washing) and dried at 60 °C overnight. The protein content of the whole cells was measured after cell disruption (Hippe et al. 1979) by a dye binding method (Bradford 1976; Chiapelli et al. 1979). Cells contained about 60% protein related to dry weight.

Chemicals. All the chemicals used were of the highest purity available, i.e., generally of analytical grade.

The redox resin 'Serdoxit' was obtained from Serva (Heidelberg, FRG). It is a polystyrene (0.4–0.8 mm ϕ) with indigodisulfonic acid as the reducing group ($E_0' \approx -80$ mV), and was prereduced with the following solution: 1 m*M* Na-phosphate (pH = 7.2), 0.75 m*M* Ti(III)-citrate, 1 m*M* $Na_2S \times 9H_2O$. When the dependency of growth on sulphur was investigated Na_2S was omitted during the reduction of 'Serdoxit'.

FeS was prepared by mixing solutions of $Fe(SO_4)_2$ and Na_2S, washing with H_2O and drying at 105 °C. The L-methionine was obtained from Merck, Darmstadt, FRG and the Imidazole (Merck, Darmstadt) was purified by charcoal. All the gases used (Messer Griesheim, Duisburg, FRG) had a purity greater than 99.9995% (v/v). The argon contained 1.4–1.7 vppm oxygen, approximately 1.2 vppm H_2O and 2.4 vppm N_2.

Results

Attempts to define the optimal sulphur requirement for growth of *Methanosarcina barkeri* were carried out in a yeast extract and Trypticase free defined medium containing methanol as the carbon and energy source and imidazole-HCl as the buffer system. It was demonstrated that this buffer, up to a concentration of 40 m*M*, had no influence on the growth yields or the methanogenesis.

As shown in Fig. 1 minimal gas production and growth of cells (the gas production data were parallel to the dry weight data) occurred when sulphide was omitted from the growth medium. L-cysteine, which was present in the culture medium (0.85 m*M*) could not serve as an alterna-

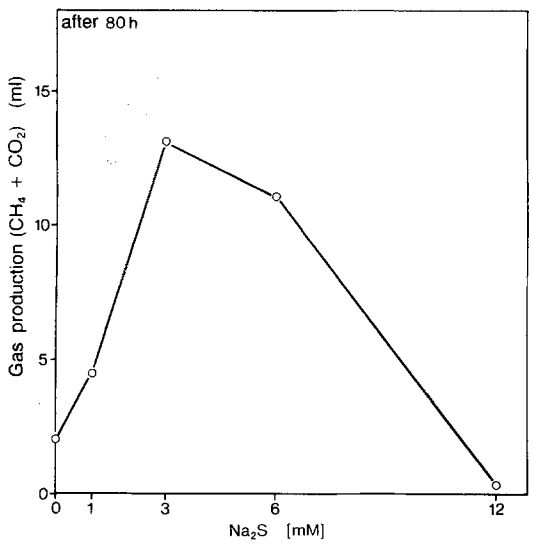

Fig. 1. Effect of various sodium sulphide concentrations on the methanogenesis of *Methanosarcina barkeri*. After autoclaving the following concentrations of $HS^- + S^{2-}$ were determined: $6 \times 10^{6-}$ M (1 m*M* Na_2S added); 5×10^{-5} M (3 m*M* Na_2S added); 4×10^{-4} M (6 m*M* Na_2S added) and 9.5×10^{-4} M (12 m*M* Na_2S added). The following growth yields (g dry weight/mol methanol consumed were obtained: Y_{MeOH} = 3.1 (1 m*M* Na_2S added), Y_{MeOH} = 4.1 (3 m*M* Na_2S added) and Y_{MeOH} = 4.2 (6 m*M* Na_2S added). Sealed culture tubes with 5 ml medium containing methanol were used. Growth temperature was 30 °C at a pH of 6.35

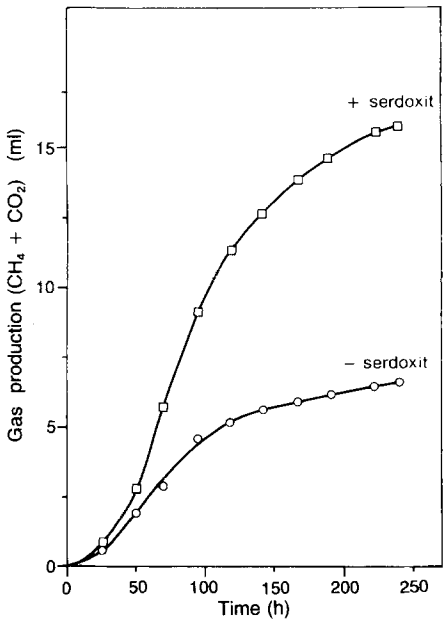

Fig. 2. Influence of the addition of the redox resin 'Serdoxit' (15 mg dry weight/5 ml) on methanogenesis of *Methanosarcina barkeri*. The culture conditions were the same as in Fig. 1 but only 0.1 m*M* Na_2S was added to the culture medium

tive to sulphide in restoring growth. Furthermore the redox potential of the medium was sufficiently reduced, due the addition of titanium citrate (Zehnder and Wuhrmann 1976), for the growth of *M. barkeri*, thus indicating that the requirement for sulphide could not have been merely a reducing agent. To determine the level of sulphide giving optimum growth, sulphide was added to the culture media at different concentrations (in the range 0 m*M* to 12 m*M*). After 80 h of incubation gas production reached a maximum at 3 m*M* sodium sulphide and declined very rapidly at higher levels, a concentration of 12 m*M* caused nearly complete inhibition (Fig. 1). However, it must be mentioned that after initial readjustment of the pH value of the culture medium and after autoclaving, the actual sulphide concentration in the medium was much lower than the amount added, since by these treatments a considerable amount of the sulphide was converted into H_2S.

However since the experiments were carried out in closed culture tubes, the H_2S was still available as a sulphur source. When only small amounts of sulphide were added to the culture medium (0.1 m*M* Na_2S) the addition of the redox resin 'Serdoxit' (15 mg dry weight/5 ml) caused a significant stimulation of growth (Fig. 2). Since similar effects were not obtained by increasing the concentration of titanium citrate in the medium this phenomenon can be explained by the fact that 'Serdoxit' is not only a redox resin, but also a weak ionic exchange resin which can bind approximately 0.4 μg $HS^- + S^{2-}$/mg dry weight under the described conditions. Furthermore it could be demonstrated that apart from 'Serdoxit' some other reduced polymeric ionic exchange resins such as Amberlite XAD-2 or XAD-7 were able to act as a kind of sulphide reservoir. Since *M. barkeri* usually forms cell aggregates which settle at the bottom of the culture tubes, there might be also an effect of a microenvironment between the microorganisms and the resin; such phenomena have been discussed since the early days of the cultivation of methanogens (Breden and Buswell 1933). Subsequently 'Serdoxit' was used as an additive because it facilitated the growth of *M. barkeri*.

Figure 3 shows that iron sulphide (solubility product approximately 10^{-19}) can also be used as a sulphur source by *M. barkeri* during growth on methanol. To heighten the influence on growth, FeS was mortared before use. The addition of 200 mg ZnS/5 ml with a solubility product of 10^{-24}–10^{-26} (D'Ans-Lax 1967) was also effective in restoring the ability of the culture to grow on methanol without the addition of sodium sulphide, but growth yields were somewhat lower under these conditions. The results demonstrate that *M. barkeri* needs only a low stationary concentration of sulphide for optimal growth. Furthermore it was observed that cells grown in the presence of 20 mg FeS/5 ml showed a signifi-

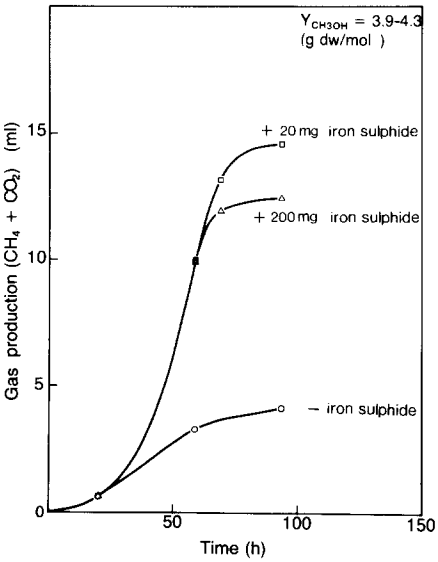

Fig. 3. Influence of various iron sulphide concentrations on the methanogenesis of *Methanosarcina barkeri* during growth on methanol. The conditions were the same as in Fig. 2, but no Na_2S was added

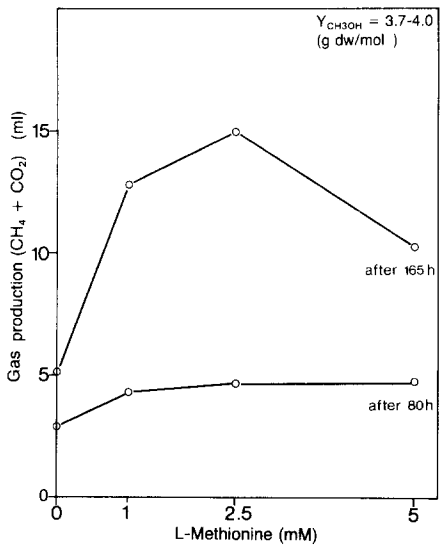

Fig. 4. Influence of various L-methionine concentrations on the methanogenesis of *Methanosarcina barkeri* during growth on methanol. Culture conditions as in Fig. 2, but no Na_2S was added

cantly increased blue green fluorescence. Previous studies have shown that all methanogens contain a blue green fluorescent coenzyme, Factor F_{420} (Eirich et al. 1979); therefore this effect could be explained by an increased formation of this Factor F_{420} under the above conditions because protein contents were comparable to controls. However, in such FeS-medium the lysis of *M. barkeri* after growth occurred more rapidly.

In anaerobic microbial ecosystems with sulphate present there is increasing evidence that on the one hand methanogenesis is inhibited by the presence of suphate-reducing bacteria, which compete for methanogenic substrates such as hydrogen or acetate, and on the other hand the methanogens are dependent on the production of sulphide (Mah et al. 1977; Zeikus 1977; Zehnder 1978). Minimal gas production was obtained from *M. barkeri* in a methanol containing medium in which sulphide was replaced by sulphate, but in a coculture with the sulphate reducing bacterium *Desulfovibrio gigas* (DSM 496) growth was restored (Scherer and Sahm 1979; Zhilina and Zavarzin 1973). When the influence of ferrous ammonium sulphate was tested on the growth and gas production of *M. barkeri* in the usual sulphide containing medium neither a stimulatory nor an inhibitory effect could be observed at concentrations of 10^{-3} and 10^{-2} M.

It has recently been demonstrated that the growth of *M. barkeri* is stimulated by the sulphur containing amino acid L-cysteine in the presence of Na_2S (Scherer and Sahm 1979, 1980). In this case the second sulphur containing amino acid L-methionine can be used instead of Na_2S as the sulphur source. Figure 4 shows that after 165 h of growth the gas production and cell dry weight reached a slight maximum at 2.5 m*M* methionine. However, the growth rate and the gas production rate were slower with L-methionine than with sodium sulphide (Fig. 1) indicating that this amino acid is not so efficient as Na_2S as a sulphur source.

Figure 5 shows a typical growth curve for *M. barkeri* in the improved medium with methanol as substrate. Cell protein rose parallel to methane formation and during growth no cell lysis was detected by microscopical studies and measurements of protein in the culture medium. A specific growth rate of 0.08–0.10 h^{-1} (corresponding to a doubling time of 7–9 h) was observed during the exponential growth phase. In comparison Mountfort and Asher described a doubling time of 64 h for *M. barkeri* when yeast extract and Trypticase were omitted from the culture medium. The maximum growth yield was approximately 0.14 g (dry weight) cell per g substrate consumed.

Many workers have experienced difficulty in obtaining growth of *M. barkeri* on acetate medium using an inoculum of cells pregrown on methanol or H_2–CO_2 (Khan 1980; Schnellen 1947; Smith and Mah 1978; Winter and Wolfe 1979). Lags of over a month have been observed for such transfers, and sometimes they failed to grow at all. Smith and Mah (1978) found that in the presence of 0.03% $Na_2S \times 9H_2O$ (pH = 7) cell lysis occurs at the end of methanol utilization which may be responsible for this long lag period. Therefore the behaviour of *M. barkeri* towards acetate-methanol mixtures was examined under the optimized sulphide concentration by inoculating

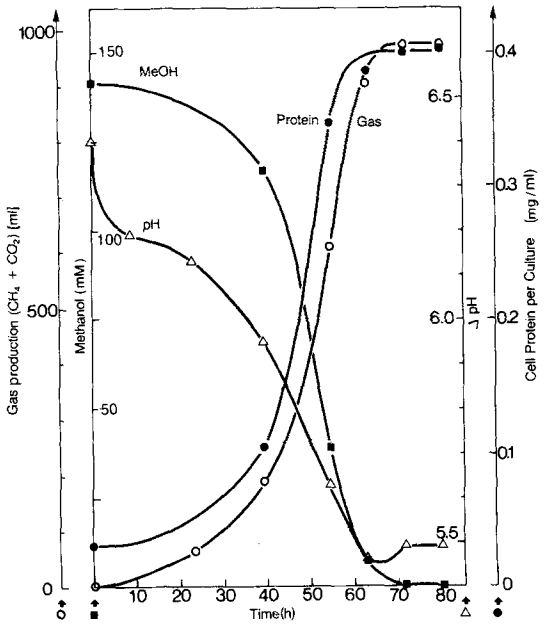

Fig. 5. Growth and methane production of *Methanosarcina barkeri* in a defined medium on methanol with 3.75 m*M* Na_2S. The culture vessel contained 350 ml medium and had a gas otulet. The medium was stirred by a magnetic stirring bar at about 60 rpm. Other culture conditions as in Fig. 1

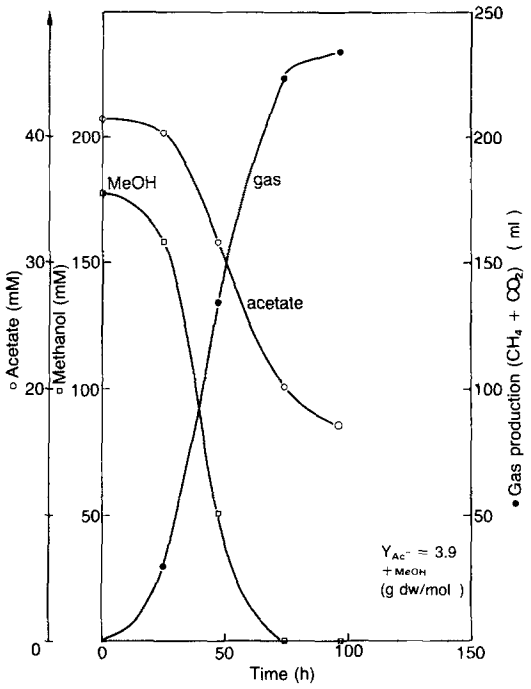

Fig. 6. Growth of *Methanosarcina barkeri* on a substrate mixture composed of 175 m*M* methanol and 42 m*M* Na-acetate. Sealed culture vessels with 50 ml medium and 2.5 m*M* Na_2S were used. Growth temperature 30 °C. Other conditions as in Fig. 2

2.5 ml of a methanol-grown culture into 50 ml of the defined medium containing 175 m*M* methanol, 42 m*M* sodium acetate and 2.5 m*M* Na_2S in 250 ml sealed culture flasks. The results (Fig. 6) show that methanol and acetate were metabolized simultaneously without any significant lag phase. Under these conditions methanogenesis was biphasic. During the first phase (75 h) methanol and acetate were utilized together in the course of which methanol was the preferred methanogenic substrate. Once the methanol was depleted, methanogenesis occurred at a much slower rate with acetate alone serving as the methanogenic substrate.

Although in recent years it has been demonstrated that pure cultures of *M. barkeri* are able to grow on acetate and to form methane by decarboxylation of acetate (Mah et al. 1978; Smith and Mah 1978; Zhilina 1978; Scherer and Sahm 1979, 1980; Winter and Wolfe 1979; Zinder and Mah 1979; Mah et al. 1980; Smith and Mah 1980), the notion that supplementary energy sources are required for growth and for methanogenesis from acetate has persisted because of thermodynamic and mechanistic considerations (Smith et al. 1980; Thauer et al. 1977). As shown in Fig. 7 *M. barkeri* can grow on sodium acetate as the source of organic carbon and energy under optimal sulphide conditions with an inoculum culture grown on methanol (methanol was completely consumed and more than 6 growth periods cultivated on methanol). However, methanogenesis by cultures growing on acetate usually ceased before the substrate was completely consumed, most likely due to a change of the pH in the medium. During growth on Na-acetate the pH value in the culture medium increases and since the growth rate of *M. barkeri* is very much reduced at pH values above 7.0, the medium should have some buffer capacity (e.g., 40 m*M* imidazole or 25 m*M* phosphate buffer) or the pH should be corrected by the addition of acid (Fig. 7). Under these latter conditions sodium acetate was metabolized until no acetate could be detected in the medium ($\leqslant$ 0.2 m*M*). The rate of acetate consumption was the same as the rate of gas (CH_4 + CO_2) formation. During the exponential growth phase a maximum specific growth rate of 0.02 h^{-1} (corresponding to a doubling time of 30 h) was observed. The growth yields were 0.05–0.07 g (dry weight) cells per g acetate (Ac^-) consumed.

It was demonstrated that not only *M. barkeri* strain DSM 804, but also the strains DSM 800, DSM 805 and DSM 1232 are able to grow on sodium acetate without a significant lag phase, although the inocula are always grown on methanol. Furthermore it was shown for *M. barkeri* DSM 804 that growth on Na-acetate was possible without a significant lag phase when the inoculum culture was grown on H_2–CO_2, but this growth was strongly retarded by the presence of H_2 (Smith and Mah 1978; Weimer and Zeikus 1978; Mah et al. 1980).

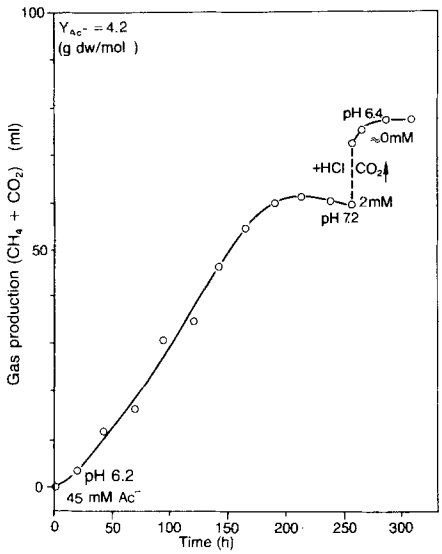

Fig. 7. Growth of *Methanosarcina barkeri* on Na-acetate. A sealed culture vessel with 50 ml medium and 2.5 m*M* Na_2S was used. Other conditions as in Fig. 2

Discussion

The results indicate that the presence of sulphide in a defined medium is essential for the growth and methanogenesis of *Methanosarcina barkeri* DSM 804. The sulphide requirement could not have been merely as a reducing agent since the redox potential was low enough to permit the growth of methanogenic bacteria when adding titanium(III)-citrate (Zehnder and Wuhrmann 1976). A similar requirement for sulphide has been shown for other methanogenic bacteria. Some 40 years ago Barker (1940) observed that minimal growth of *Methanobacterium omelianskii* occurred when sulphide was omitted from the growth medium. Bryant et al. (1971) showed that in cultures of *Methanobrevibacter smithii* and *Methanobacterium bryantii* sulphide acted as a sulphur source. Furthermore it was found for *Methanobacterium* strain AZ that the addition of sulphide, – besides cysteine as a sulphur source –, caused a remarkable stimulation of growth (Wellinger and Wuhrmann 1977).

M. barkeri DSM 804 was also able to grow on methanol in a defined medium supplemented with L-cysteine and L-methionine in the absence of added sulphide. So far L-methionine has not been described as a sulphur source for methanogens. Recently Mah et al. (1978) reported that *M. barkeri* strain 227 could be grown in yeast extract medium without adding sulphide.

Inhibitory effects of high levels of sulphide on methanogenesis in ecosystems are well known (Mah et al. 1977; Zeikus 1977; Zehnder 1978). However, it is not known whether the toxicity of sulphide is direct or indirect or both. Thus sulphide could exert an indirect effect by removing the metal ions essential for growth of the methanogenic bacteria. Recently, Mountfort and Asher (1979) reported that the specific growth rate of *M. barkeri* strain DM (ATCC 29824) was decreased at a high sulphide level (12.5 m*M* added) whereas the specific rate of methane production was increased. They were able to demonstrate that cellular ATP levels declined when cells were grown at higher sulphide levels. This suggests that sulphide may have a direct effect on the metabolism by uncoupling ATP production from methane formation. This assumption is also supported by the finding that methanogenesis by resting cells of *M. barkeri* DSM 804 incubated in a buffer solution is inhibited by sulphide (unpublished results).

The results reported here indicate that the presence of only very low stationary sulphide concentrations are necessary for the growth and methanogenesis of *M. barkeri* DSM 804. Therefore this strain cannot only use sodium sulphide but also iron sulphide or zinc sulphide, which have a very low solubility, as sulphur source. Although the addition of 'Serdoxit' caused a stimulation during growth on these sulphur compounds, control experiments showed that 'Serdoxit' is not essential for their utilization.

Furthermore the necessity for only low sulphide concentrations for growth of *M. barkeri* DSM 804 was also demonstrated by batch cultures growing on a mixture of methanol and acetate in which a constant sulphide level was adjusted by a sulphide sensitive electrode. The resulting optimal stationary concentration was below 0.005 m*M* dissolved S ($HS^- + S^{2-}$) leading to a $\mu_{max} \leqslant 0.14\ h^{-1}$ ($t_d \leqslant 5$ h) (unpublished results). This is in accordance with the results of Cappenberg (1975) achieved with a *Methanobacterium* (presumably *M. söhngenii*) growing on acetate. The growth of this isolate was inhibited by a constant concentration of 0.1 m*M* hydrogen sulphide whereas a concentration of 0.001 m*M* or lower was found to be optimal.

When methanol and acetate were both added to the growth medium, both compounds were metabolized simultaneously. Similar results were also obtained with methyl acetate after its slow partial hydrolysis to methanol and acetate at a pH value of about 6.5 of the medium. A simultaneous conversion of methanol and acetate has been described previously for the thermophilic *Methanosarcina* strain TM-1 (Zinder and Mah 1979; Smith et al. 1980). Using radioactive labelled substances it was found that for this strain under these conditions acetate was largely incorporated into cell mass or oxidized and the electrons derived from this process were used apparently to reduce methanol to methane. However it has been found by Smith and Mah (1978) and Mah et al. (1980) that the growth curve of the mesophilic *Methanosarcina* strain 227 on methanol-acetate mixtures resembles a diauxic growth curve, i.e., methanol and acetate were utilized sequentially. Similar diauxic growth

responses with *M. barkeri* were obtained by Schnellen (1947). The diauxic growth effect described by Smith and Mah (1978) was influenced in some way by the concentration of sodium sulphide in the culture medium. Therefore it seems that the capability of *Methanosarcina* to utilize methanol and acetate simultaneously for growth and methanogenesis is probably dependent on the strain and the culture conditions.

Since the standard-free energy change for the decarboxylation of acetate to methane and carbon dioxide is low ($\Delta G'_0 = -31.0$ kJ/mol) compared with the standard-free energy change for the hydrolysis of ATP ($\Delta G'_0 = -31.8$ kJ/mol) (Thauer et al. 1977), investigators have assumed that the utilization of acetate might be attributed to cometabolism, and energy for growth may come instead from other substrates in the culture medium. However, the results reported here rule out this cometabolism-hypothesis for *M. barkeri* and indicate that the capability of strains of *Methanosarcina* to utilize acetate as the sole source of carbon and energy is probably widespread (Zhilina 1978; Smith and Mah 1980; Smith et al. 1980). The failure of previous workers to grow different *Methanosarcina* species on mineral acetate medium and to show a complete conversion of acetate may be related to the lack of a sulphide reservoir and maintenance of a low oxidation-reduction potential, which organic compounds may provide in a complex medium. It seems that the sulphide concentration and the pH value are very important in cultivating these bacteria on acetate. Since Oppermann et al. (1957) and Hansson (1979) have observed that methanogenesis from acetate in mixed cultures is inhibited by CO_2, the influence of the pH value may be indirect by changing the concentration of dissolved CO_2 in the medium.

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Note Added in Proof

The presented results that acetate and methanol can be used simultaneously (Weimer and Zeikus 1978) during growth were recently confirmed by Hutten et al. (Antonie van Leeuwenhoek, J Microbiol Serol, in press) with the mesophilic *M. barkeri* culture DSM 800 (=strain 'MS').

Further experiments with a pH and a sulphide potential controlled 6 l continuous culture of *M. barkeri* DSM 804 growing either on Na-acetate (100 m*M*) or on methanol (180 m*M*) showed that for similar gas production rates (e.g., 50 ml/l/h) the sulphide addition during growth on acetate as sole substrate was at least 10 fold lower.