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Conversion of Glucose to Fatty Acids and Methane: Roles of Two Mycoplasmal Agents

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Two species of obligately anaerobic mycoplasmas were the major components of a methanogenic glucose-limited enrichment culture. In pure culture, one of these organisms, tentatively named *Anaeroplasma* sp. strain London, was shown to be responsible for the fermentation of glucose to fatty acids, hydrogen, and carbon dioxide; the other mycoplasma was shown to produce methane from hydrogen and carbon dioxide and was named *Methanoplasma elizabethii*. This same methanogenic mycoplasma contained a low-molecular-weight fluorescent cofactor which had a maximum light absorbance at 430 nm. When both species of mycoplasmas were grown together on glucose, fermentation products included fatty acids and methane. For the first time, mycoplasmas are implicated as agents of anaerobic degradation and methanogenesis in a sewage sludge digester.

In anaerobic sludge digestion, organic matter is degraded to methane and carbon dioxide as a result of the combined metabolic activities of the microbial population. The steps involved in the breakdown of carbohydrate to carbon dioxide and methane in the sludge digester are little understood, although fatty acids, such as butyric and acetic acids, have been shown to be intermediates in this process (20).

Anaerobic mycoplasmas have been isolated from habitats where methane is produced (7, 15), but knowledge of their role in anaerobic digestion has remained obscure. A methanogenic enrichment culture, isolated after inoculation of a glucose-limited chemostat with fluid from a sewage sludge digester, was found to consist largely of mycoplasmal agents (17). The present study was undertaken to isolate these species of mycoplasma in pure culture and to establish their roles in the fermentation of glucose to methane. Two different species of mycoplasmas were identified, one converting glucose to fatty acids, hydrogen, and carbon dioxide, and the other converting carbon dioxide and hydrogen to methane. The work presented here indicates that mycoplasmas could be significant agents of natural anaerobic fermentation.

MATERIALS AND METHODS

Culture methods. Anaerobic techniques based on the fundamental techniques of Hungate (4, 6) were used throughout the investigation. The use of an anaerobic cabinet (Heinicke National Appliance Co., model 3615) permitted microbiological techniques to be performed in a controlled anaerobic environment. Loops were sterilized in the cabinet by means of an electric sterilizer (Bact-Cinerator, Sherwood).

Culture media. Mineral medium (18) contained (grams per liter): NH_4Cl, 2.2; KH_2PO_4, 1.13; $NaH_2PO_4 \cdot 2H_2O$, 1.6; $Na_2HPO_4 \cdot 2H_2O$, 7.12; $MgSO_4 \cdot 7H_2O$, 0.23; $CaCl_2 \cdot 2H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.014; $MnSO_4 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, 0.002; $CuSO_4 \cdot 5H_2O$, 0.0004; $CoCl_2 \cdot 6H_2O$, 0.0004; EDTA (disodium salt), 0.081; resazurin, 0.001; and $Na_2S \cdot 9H_2O$, 0.25.

The growth factor supplement consisted of (milligrams per liter): folic acid, 0.02; guanine, 0.01; riboflavin, 0.1; nicotinic acid, 0.3; nicotinamide, 0.2; inositol, 0.1; thiamine hydrochloride, 0.1; pantothenic acid, 0.1; pyridoxine, 3.0; and biotin, 0.01.

The pH of each medium was adjusted to pH 7.2 by the addition of 2 M NaOH.

Medium A consisted of mineral medium, growth factor supplement, glucose (2.5 mM), and yeast extract (Difco Laboratories; 0.5%). Cultures were incubated under nitrogen in this medium. Medium B consisted of mineral medium, growth factor supplement, and yeast extract (0.5%). Cultures were incubated under 80% hydrogen and 20% carbon dioxide in this medium. The media were solidified for plate cultures by the addition of agar to a concentration of 1.7% (wt/vol).

Penicillin, when required, was added aseptically to give a final concentration of 5,000 U ml^{-1}.

Colony isolation. Mineral medium was sterilized, reduced, dispensed into test tubes, sealed, and transferred to the anaerobic cabinet. Microbial culture was serially diluted by syringe, and the required dilution was inoculated onto the agar medium.

Molten sterile agar medium was cooled under nitrogen to 40°C, transferred to the anaerobic cabinet, and distributed into petri dishes (medium A) or as slopes in narrow-necked McCartney bottles. In the latter case, the gaseous atmosphere of nitrogen was replaced by H_2-CO_2 (80:20). Cultures were plated on the surface of the agar.

Batch culture. Culture medium was sterilized and reduced in 250-ml conical flasks. A 10-ml amount of medium was anaerobically transferred to sterile test tubes, flushed with nitrogen or hydrogen-carbon dioxide, and sealed with butyl rubber stoppers. Inoculation of colonies, or subculture of existing cultures was performed in the anaerobic cabinet. Batch cultures were incubated at 37°C without shaking.

Analytical techniques. Optical densities of samples were determined with a light path of approximately 1 cm in a colorimeter (Evans Electroselenium Ltd., Halstead, Essex, U. K.) equipped with a green filter. Distilled water was used as the blank. A reading of 0.25 corresponded to an approximate dry weight of 0.1 g liter^{-1}.

Dry weights of cultures were determined in triplicate. Cells were centrifuged, washed twice with distilled water, and dried at 105°C to constant weight.

Analyses of butyric, propionic, and acetic acids were performed on 1-μl samples, acidified with 85% phosphoric acid, and injected into a glass column (6 ft by 0.25 in.; 183.0 by 0.6 cm) containing Chromosorb century series 101, 100/120 mesh (Johns-Manville, Denver, Colo.) held at 200°C in a Pye 104 gas chromatograph equipped with a flame ionization detector. The carrier gas was nitrogen at a flow rate of 50 ml min^{-1}; both samples and standards were analyzed in triplicate. Gaseous end products were analyzed by the method of Nelson and Zeikus (9).

Glucose was measured by the glucose oxidase method (Biochemica Test Combination, Boehringer Mannheim Corp.).

Fluorescent cofactor. Low-molecular-weight fluorescent cofactor was extracted by a procedure supplied by L. Doddema (Katholeike University, Nijmegen, Holland, personal communication). Culture samples (100 ml, containing approximately 0.07 g [dry weight] of cells) were heated at 100°C for 10 min and spun at 9,000 × *g* for 10 min at 4°C. The resulting supernatant was diluted with 1 volume of propanol and spun at 9,000 × *g* for 10 min. The final supernatant was made alkaline by the addition of 0.1 volume of potassium hydroxide (2 M).

Absorbance was measured in a Pye Unicam SP 500 series 2 spectrophotometer, and fluorescence was measured in a Locarte fluorimeter, with a 50:50 propanol-water mixture being used as the blank in both cases. Samples were reduced by the addition of sodium dithionite to fluorescence cuvettes. For the fluorescence (excitation) spectrum, the exciting wavelength was 340 nm.

Light microscopy. Photographs were produced with a Nikon microscope fitted with phase optics. Phase-contrast photomicrographs were made with shutter speeds of 5 to 8 s on Ilford Ilfordata HS 23 microfilm with an ASA rating of 12.

Electron Microscopy. Culture samples were fixed in glutaraldehyde fixative (3% glutaraldehyde in mineral medium) for 1 h and centrifuged. The fixed pellet was washed several times over 2 h at room temperature with 0.2 M phosphate buffer and centrifuged. After post-fixing in 1% osmium tetroxide for 1 h, culture pellets were dehydrated by passage through increasingly concentrated ethanol. Samples were then placed in Spurs resin and rotated overnight, followed by polymerization. Embedded material was sectioned and observed under an AEEM 6B electron microscope.

RESULTS

Enrichment culture and isolation of pure cultures. An anaerobic chemostat culture containing glucose and mineral medium was inoculated with digester sludge (taken from the Mogden Plant, Twickenham, London) and maintained at pH 7.2 by automatic addition of 2 M NaOH. A stable glucose-limited culture developed which fermented glucose to fatty acids, methane, and carbon dioxide (17). An electron micrograph of this culture is shown in Fig. 1. The most prominent and numerous organisms in the culture were identified as mycoplasmas by their lack of typical bacterial cell walls. The organisms present in this culture were isolated and identified as (i) four species of facultative anaerobic bacteria, present at a low concentration (2×10^5 organisms ml^{-1}) and identified as *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Proteus mirabilis*; (ii) an obligate anaerobic species of mycoplasma, which was the most numerous species (10^{11}, organisms ml^{-1}), and (iii) an obligate anaerobic species of mycoplasma, present at low concentration in the anaerobic glucose-limited chemostat (less than 10^6 organisms ml^{-1}) and found to be capable of producing methane.

The enrichment culture was plated in the anaerobic cabinet onto medium A (under a gaseous atmosphere of nitrogen) or medium B (under a gaseous atmosphere of hydrogen and carbon dioxide). The most numerous colonies grown on medium A were isolated in pure culture. Surface colonies of this organism were white and umbonate (that is, "fried egg" morphology) and reached a diameter of 1 to 3 mm after 48 h of incubation. This organism has tentatively been classified as an *Anaeroplasma* sp. (14) and termed strain London.

The organism responsible for the production of methane in the glucose-limited enrichment culture was also found to be an obligate anaerobic species of mycoplasma. Colonies of this organism, on medium B, were yellow and umbonate and reached a diameter of approximately 100 μm after 72 h of incubation. Upon exposure to air, these colonies turned brown.

The novelty and significance of methane production by a species of mycoplasmas justified inclusion into a new genus. The methanogenic mycoplasma has consequently been named *Methanoplasma elizabethii*.

Properties of *Anaeroplasma* sp. strain London. Despite growth on mineral medium and glucose in the enrichment culture, growth

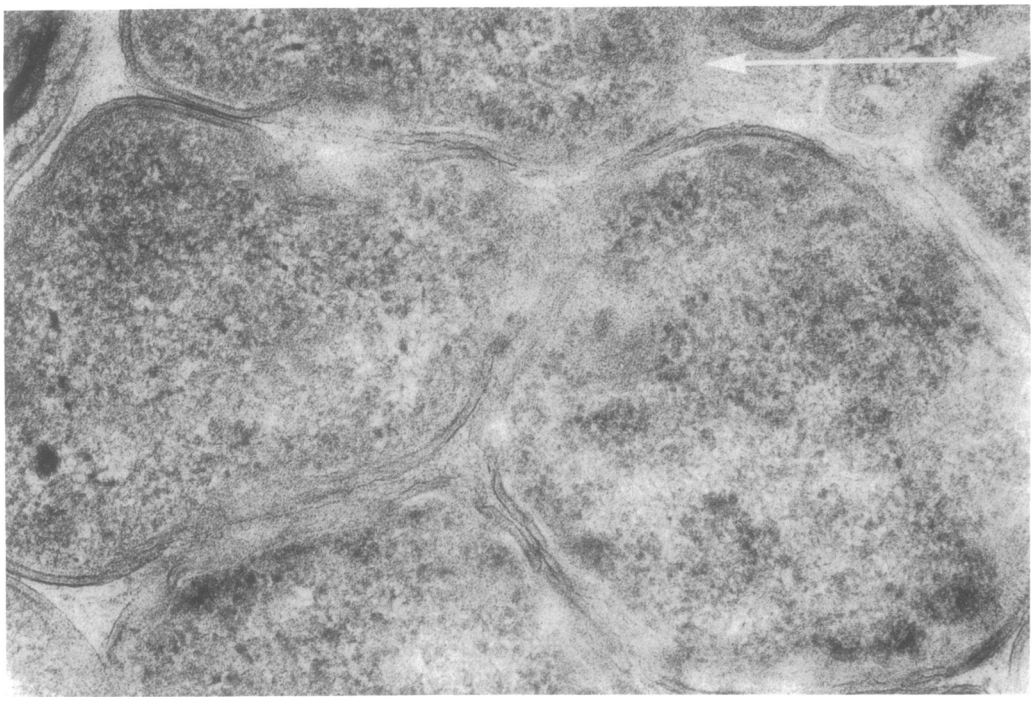

FIG. 1. *Thin-section electron micrograph of a glucose-limited methanogenic enrichment culture. Bar marker, 0.5 μm.*

of *Anaeroplasma* sp. strain London was not obtained in pure culture without the presence of both yeast extract and growth factor supplement. The presence of this latter requirement removed the obligate growth requirement for serum that had been previously observed (17). Growth was not obtained on medium A with the omission of glucose.

The Gram-stained culture appeared as unrecognizable debris under the light microscope. However, recognizable morphology was apparent by phase-contrast microscopy of an unstained preparation. Figure 2 is a phase-contrast photomicrograph of a culture of *Anaeroplasma* sp. strain London in exponential growth. The individual cells were spindle shaped and showed branching. The individual cells were connected by thin filaments to form short chains. Figure 3 gives the growth curve for the culture; the minimum doubling time was 20.4 h. The dry weight at stationary phase was 0.1 g dry weight liter^{-1}, giving a molar growth yield of 40 g (dry weight) per mol of glucose. Table 1 gives the carbon balance for this fermentation. Biomass carbon was calculated, assuming that 48% of the dry weight was carbon. Glucose consumed for energy is assumed to be the total glucose minus the glucose required to provide cell carbon. The results indicate that almost 1 mol of butyrate, 2 mol of hydrogen, and 1.5 mol of carbon dioxide were produced per mol of glucose consumed for energy.

Growth of *Anaeroplasma* sp. strain London was unaffected by the presence of penicillin G at 5,000 U ml^{-1}. This organism possessed the ability to pass through a membrane filter (0.45 μm; Millipore Corp.). This ability was observed by examination of the filtrates under phase contrast microscopy. Also, culture was forced by syringe through a membrane filter of 0.22-μm pore size, and viable counts showed the presence of 5×10^3 colony-forming units ml^{-1} in the filtrate compared with 10^{10} colony-forming units ml^{-1} in the unfiltered material. The low viable count of the filtrate could reflect either difficulty in maintaining strict anaerobiosis or damage to the cells caused by shearing during the filtration rather than lack of filterability.

Properties of *M. elizabethii*. Growth of *M. elizabethii* was obtained by inoculation into medium B under a gaseous atmosphere of hydrogen and carbon dioxide. Colonies were also inoculated into control tubes containing a nitrogen atmosphere; growth and methane production were not observed in these tubes. The *Methanoplasma* species grew on formate (20 mM) in medium B and produced methane. It failed to grow on acetate (20 mM) in medium B over a

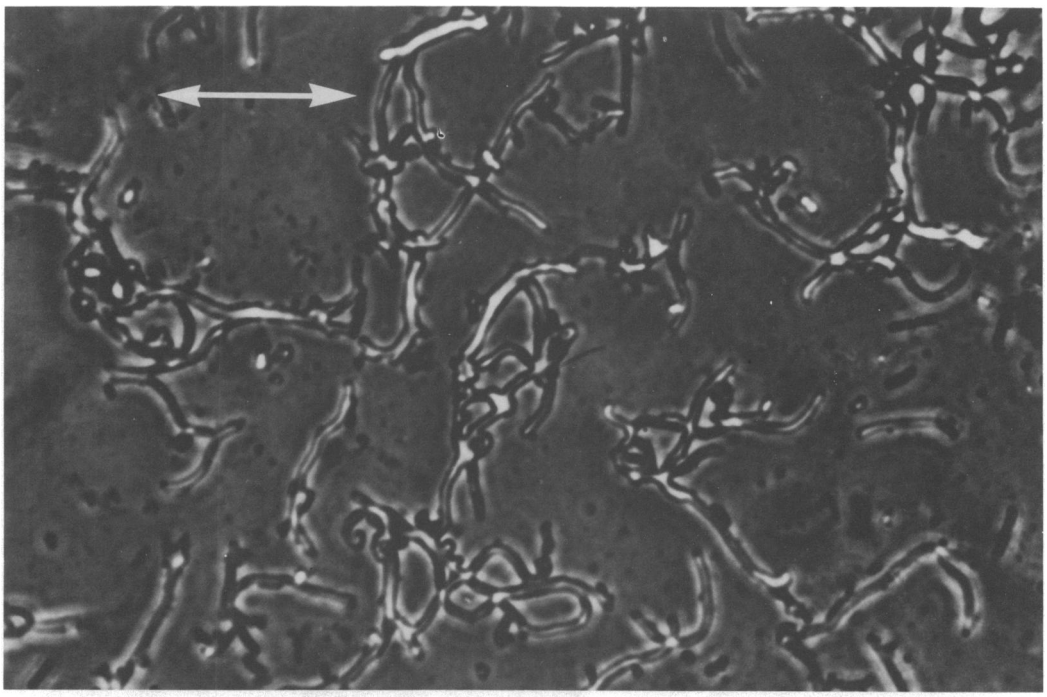

FIG. 2. *Phase-contrast photomicrograph of a pure culture of Anaeroplasma sp. strain London grown on medium A. Bar marker, 5 μm.*

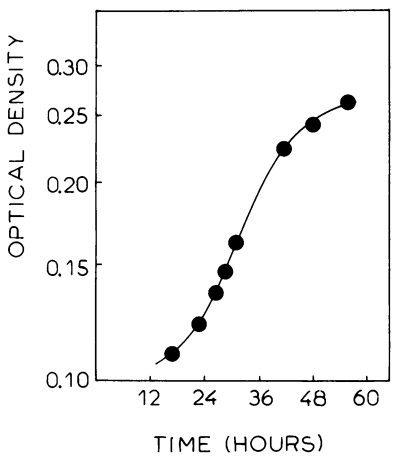

FIG. 3. *Growth curve of a pure culture of Anaeroplasma sp. strain London in batch culture in medium A.*

40-day period.

Figure 4 is a phase-contrast photomicrograph of a pure culture of *M. elizabethii* taken when the culture was in exponential growth. Figure 5 gives the growth curve for the pure culture; the minimum doubling time was 43 h, and the specific rate of methane production (q_p) in medium B was 1.68 mmol g (dry weight)$^{-1}$ h^{-1}, giving a molar growth yield ($Y = \mu/q_p$) of 9.5 g (dry weight) per mol of methane.

Growth and methanogenesis by *M. elizabethii* were unaffected by the presence of penicillin G

TABLE 1. *Product balance for the fermentation of glucose by a pure culture of Anaeroplasma sp. strain London in medium A under nitrogen*[a]

Product	Amt (mol) of product per 100 mol of glucose from:	
	Total glucose consumed	Glucose consumed for energy[b]
Butyric acid	70.3	95.9
Propionic acid	3.1	4.2
Acetic acid	0.0	0.0
Hydrogen	142.3	194.0
Carbon dioxide[c]	123.0	167.7
Methane	0.0	0.0
Biomass carbon	160.0	
Carbon recovery		95.6%
Oxidation/reduction balance		0.86

[a] Incubation time was about 60 h; all of the glucose was consumed.

[b] Total glucose minus glucose required to provide biomass carbon, that is, (100 − 160/6)% of the glucose.

[c] Value represents gaseous plus dissolved (calculated) carbon dioxide.

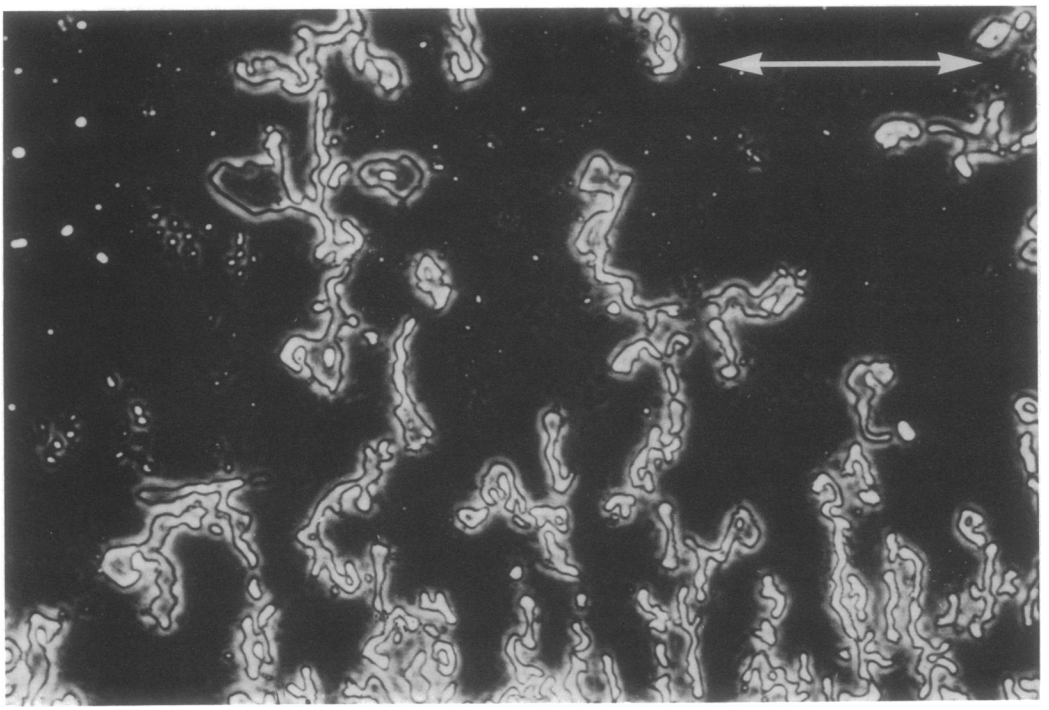

FIG. 4. *Phase-contrast photomicrograph of a pure culture of M. elizabethii grown on medium B. Bar marker, 5 μm.*

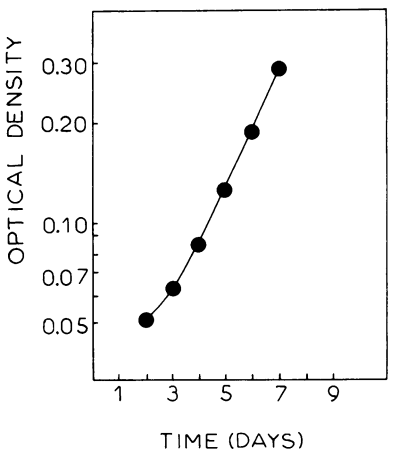

FIG. 5. *Growth curve of a pure culture of M. elizabethii on medium B in batch culture.*

at 5,000 U ml^{-1}. This organism also possessed the ability to pass through a 0.45-μm membrane filter.

Figures 6 and 7 give the respective absorbance and fluorescence spectra for a fluorescent cofactor isolated from a pure culture of *M. elizabethii*. The absorbance maxima occurred at a wavelength of 430 nm.

Growth of a consortium of *Anaeroplasma* strain London and *M. elizabethii* on glucose. Table 2 gives the product balance for the fermentation of glucose in the presence of both species of mycoplasma. Sufficient hydrogen would have been produced in the formation of acetyl coenzyme A from pyruvate to account for the production of methane observed by the coculture. The presence of *M. elizabethii* also permitted the production of acetate by *Anaeroplasma* sp. strain London.

DISCUSSION

Both species of mycoplasma were classified in the family *Mycoplasmataceae* on the basis of colonial morphology, microscopic morphology, ability to pass through a 0.45-μm membrane filter, and resistance to penicillin G. Despite the lack of a cell wall and apparent absence of axial filaments, most species of mycoplasma form filaments (11). The branched filaments observed for both *Anaeroplasma* sp. strain London and *M. elizabethii* are typical of mycoplasmal growth (2). Very high counts of colony-forming units of the *Anaeroplasma* sp. and *Methanoplasma* sp. (10^{11} ml^{-1}) with 1 mg (dry weight) ml^{-1} were obtained. Such high counts suggest that the cells could give rise to exceptionally

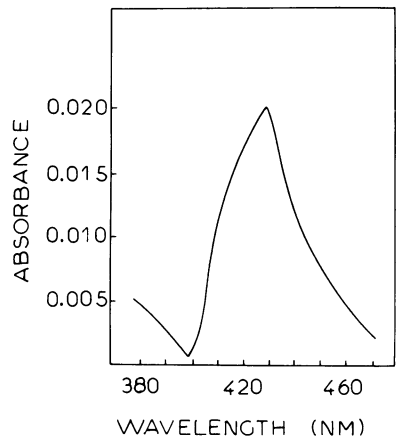

FIG. 6. *Absorbance spectrum for the cofactor isolated from a pure culture of M. elizabethii.*

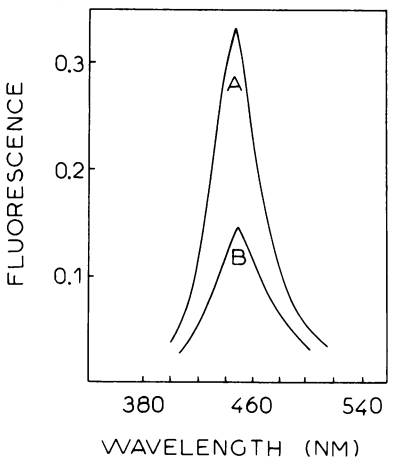

FIG. 7. *Fluorescence spectra for the cofactor isolated from a pure culture of M. elizabethii. A, Oxidized; B, partially reduced.*

small minimal reproductive units. Besides the filamentous branched cells seen under phase, there were numerous small particles near the limit of resolution of the light microscope (Fig. 2 and 4). Whether these small particles are the minimal reproductive units or debris remains a problem to be solved.

The colonies of the species of *Mycoplasmataceae* are distinctive and constitute the primary criterion for the presence of mycoplasmas. Typically umbonate in appearance, the average colony diameter of mycoplasmas is 100 μm (19); the average colony diameter of *M. elizabethii* was of this order. The average colony diameter of *Anaeroplasma* sp. strain London was larger than this, and similar to that of a species of *Anaeroplasma* isolated from the bovine rumen (15). Umbonate colonies have also been described for a species of methanogenic bacteria (16).

TABLE 2. *Product balance for the fermentation of glucose by a mixed culture of Anaeroplasma sp. strain London and M. elizabethii in medium A under nitrogen*[a]

Product	Amt (mol) of product per 100 mol of glucose from:	
	Total glucose consumed	Glucose consumed for energy[b]
Butyric acid	60.3	82.2
Propionic acid	10.9	14.9
Acetic acid	9.9	13.5
Hydrogen	0.0	0.0
Carbon dioxide[c]	84.3	114.9
Methane	33.2	45.3
Biomass carbon	169.0	
Carbon recovery		96.7%
Oxidation/reduction balance		0.85

[a] Incubation time was about 60 h; all the glucose was consumed.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.

With pure culture of *Anaeroplasma* sp. strain London, glucose was fermented primarily to butyric acid, hydrogen, and carbon dioxide. Failure to detect hydrogen when *Anaeroplasma* sp. strain London was cocultured with *M. elizabethii* was probably the consequence of rapid hydrogen assimilation by the actively methanogenic population ("interspecies hydrogen transfer" [5]).

The oxidation-reduction balance calculated from the fermentation products suggested the presence of an undetected oxidized product or products. This may, in part, be explained by the difficulty of carbon dioxide analysis. Carbon dioxide did not produce a linear thermal conductivity-concentration isotherm, and for this reason experimental error may have been greater for carbon dioxide analysis, as observed elsewhere (12).

For methanogenic bacteria, molar growth yields of between 0.6 and 1.6 g (dry weight) per mol of methane have been reported (21). The growth yield of 0.62 g (dry weight) per mol of hydrogen, reported by Roberton and Wolfe (13), can be converted to a value of 2.48 g (dry weight) per mol of methane (assuming a 4:1 ratio between hydrogen uptake and methane production). Clearly, the molar growth yield reported here for *M. elizabethii* has the much higher value of 9.5 g (dry weight) per mol of methane.

Although originally thought to be a biological constant, studies have indicated that Y_{ATP} (the

dry weight of organism produced per mol of ATP) can have a value in the range of 5.9 to 32 g (dry weight) per mol of ATP (10). Even if a Y_{ATP} value as low as 5.9 g (dry weight) per mol of ATP is assumed for growth on hydrogen and carbon dioxide, the efficiency of energy conversion for this reaction for previously reported growth yields (13, 21) varies between 2.5 and 11% (corresponding to a maximum of 0.44 mol of ATP per mol of methane produced). However, the explanation for this apparent inefficiency was not forthcoming. The efficiency of energy conversion for *M. elizabethii*, using the value of Y_{ATP} of 5.9, is 40% (1.6 mol of ATP per mol of methane produced). Since normal efficiencies of energy transfer in microorganisms are between 30 and 70% (8), this represents a more realistic figure for growth and methanogenesis on hydrogen and carbon dioxide.

The fluorescent cofactor isolated from a pure culture of *M. elizabethii* was similar to a cofactor, F_{420}, isolated from methanogenic bacteria (1), in that both exhibited fluorescence in the oxidized state; fluorescence decreased as reduction took place. Although a cofactor isolated from methanogenic bacteria had an absorption maximum at 430 nm (3), it did not exhibit fluorescence, as did the cofactor isolated from *M. elizabethii*.

The ability of the combined activity of two species of mycoplasmas to degrade glucose to methane highlights the contribution that mycoplasmas may play in the overall fermentation of glucose to methane in the sludge digester. The results from this study should encourage attention to be focused on the isolation of mycoplasmal agents from methanogenic environments.

ACKNOWLEDGMENTS

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