

Growth of *Methanosarcina barkeri* (Fusaro) under Nonmethanogenic Conditions by the Fermentation of Pyruvate to Acetate: ATP Synthesis via the Mechanism of Substrate Level Phosphorylation

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A mutant of *Methanosarcina barkeri* (Fusaro) is able to grow on pyruvate as the sole carbon and energy source. During growth, pyruvate is converted to CH₄ and CO₂, and about 1.5 mol of ATP per mol of CH₄ is formed (A.-K. Bock, A. Prieger-Kraft, and P. Schönheit, Arch. Microbiol. 161:33–46, 1994). The pyruvate-utilizing mutant of *M. barkeri* could also grow on pyruvate when methanogenesis was completely inhibited by bromoethanesulfonate (BES). The mutant grew on pyruvate (80 mM) in the presence of 2 mM BES with a doubling time of about 30 h up to cell densities of about 400 mg (dry weight) of cells per liter. During growth on pyruvate, the major fermentation products were acetate and CO₂ (about 0.9 mol each per mol of pyruvate). Small amounts of acetoin, acetolactate, alanine, leucine, isoleucine, and valine were also detected. CH₄ was not formed. The molar growth yield (Y_{acetate}) was about 9 g of cells (dry weight) per mol of acetate, indicating an ATP yield of about 1 mol/mol of acetate formed. Growth on pyruvate in the presence of BES was limited; after six to eight generations, the doubling times increased and the final cell densities decreased. After 9 to 11 generations, growth stopped completely. In the presence of BES, suspensions of pyruvate-grown cells fermented pyruvate to acetate, CO₂, and H₂. CH₄ was not formed. Conversion of pyruvate to acetate, in the complete absence of methanogenesis, was coupled to ATP synthesis. Dicyclohexylcarbodiimide, an inhibitor of H⁺-translocating ATP synthase, did not inhibit ATP formation. In the presence of dicyclohexylcarbodiimide, stoichiometries of up to 0.9 mol of ATP per mol of acetate were observed. The uncoupler arsenate completely inhibited ATP synthesis, while the rates of acetate, CO₂, and H₂ formation were stimulated up to fourfold. Cell extracts of *M. barkeri* grown on pyruvate under nonmethanogenic conditions contained pyruvate:ferredoxin oxidoreductase (0.5 U/mg), phosphate acetyltransferase (12 U/mg), and acetate kinase (12 U/mg). From these data it is concluded that ATP was synthesized by substrate level phosphorylation during growth of the *M. barkeri* mutant on pyruvate in the absence of methanogenesis. This is the first report of growth of a methanogen under nonmethanogenic conditions at the expense of a fermentative energy metabolism.

Methanosarcina barkeri is metabolically one of the most versatile methanogens. It can grow on H₂-CO₂, methanol, various methylamines, and acetate as carbon and energy sources (2, 11, 14, 29). Recently, a mutant of *M. barkeri* (Fusaro) was shown to grow on pyruvate as the sole carbon and energy source (4; see also reference 20). During growth, pyruvate is converted to CO₂ and CH₄ as follows: pyruvate⁻ + H⁺ + 0.5 H₂O → 1.25 CH₄ + 1.75 CO₂ (4). From growth studies, cell suspension experiments, and enzyme measurements, the pathway of conversion of pyruvate to CH₄ and CO₂ appears to involve the following steps (4): uptake of pyruvate by passive diffusion, oxidation of pyruvate to acetyl coenzyme A (acetyl-CoA) via pyruvate:ferredoxin oxidoreductase, conversion of acetyl-CoA to CO₂ and CH₄ via the aceticlastic reactions (10, 13, 28), and reduction of CO₂ to methane with the reducing equivalents generated by pyruvate oxidation. During growth on pyruvate, ATP yields of up to 1.5 mol/mol

of CH₄ were calculated (4). ATP synthesis coupled to methanogenesis in methanogenic bacteria has been shown to proceed via the mechanism of electron transport phosphorylation (6, 18, 23).

In this study, we investigated whether *M. barkeri* is able to grow on pyruvate when methanogenesis is completely inhibited by bromoethanesulfonate (BES), an inhibitor of methyl coenzyme M reductase (9). The rationale for these experiments was based on the observation that pyruvate-grown cells of *M. barkeri* contained high levels of phosphate acetyltransferase and acetate kinase activities (see below). The conventional function of these enzymes is the activation of acetate to acetyl-CoA during growth of *M. barkeri* on acetate (for literature, see references 10 and 13). During growth on pyruvate, the reverse reactions catalyzed by phosphate acetyltransferase and acetate kinase might convert acetyl-CoA to acetate and phosphorylate ADP under conditions in which the conversion of acetyl-CoA to methane is blocked.

It is shown here that *M. barkeri* is indeed able to grow on pyruvate in the complete absence of methanogenesis. Evidence is presented that under these conditions, the organism converts pyruvate mainly to acetate and ATP is formed by the mechanism of substrate level phosphorylation.

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MATERIALS AND METHODS

Source of materials. Pyruvate was from Serva (Heidelberg, Germany), and sodium-2-BES was from Sigma (Deisenhofen, Germany). All other chemicals were, if not mentioned otherwise, reagent grade and obtained from Merck (Darmstadt, Germany). Enzymes and coenzymes were from Boehringer (Mannheim, Germany). Gases (N_2 , 5.0; H_2 , 5.0; CO_2 , 4.8) were obtained from Messer Griesheim (Berlin, Germany). Ferredoxin from *Clostridium pasteurianum* was purified as described by Schönheit et al. (25). *M. barkeri* Fusaro (DSM 804) was from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). All experiments described in this paper were performed with the pyruvate-utilizing mutant of *M. barkeri* (4).

Growth of *M. barkeri* on pyruvate. The organism was routinely grown at 37°C in anaerobic glass bottles on mineral medium containing pyruvate as the carbon and energy source, as described before (4).

Growth of *M. barkeri* on pyruvate in the presence of BES. Growth experiments were performed under anaerobic conditions in 1,240-ml closed glass bottles filled with 150 ml of mineral medium containing 80 to 90 mM pyruvate as the sole carbon and energy source. Sodium-2-BES was added from a sterile stock solution to a concentration of 3 mM before inoculation. The gas phase was 100% N_2 at 150 kPa. The medium was inoculated (20%) with late-log-phase cells of *M. barkeri* grown on pyruvate in the absence of BES and incubated at 37°C. Growth was monitored by measuring the optical density at 578 nm (ΔOD_{578}) and the increases of protein and of cell dry weight as described previously (4). Similar relationships between protein, cell dry weight, and ΔOD_{578} were observed during growth on media containing pyruvate and pyruvate-BES. For growth yield determinations, a value of 240 mg (dry weight) of cells per liter was taken to correspond to a ΔOD_{578} of 1. Samples (2 ml) from the liquid phase were analyzed for pyruvate, acetate, amino acids, acetoin, and acetolactate (see below). Samples (0.3 ml) from the gas phase were analyzed for CO_2 , CH_4 , and H_2 by gas chromatography. The amount of HCO_3^- (or H_2CO_3) in the medium was determined as CO_2 after acidification with citric acid as described previously (4).

Preparation of cell suspensions. Cells grown on pyruvate in the absence of BES were harvested anaerobically in the late log phase. After centrifugation ($9,000 \times g$ for 25 min), the cells were washed once with cold anaerobic imidazole-chloride buffer (20 mM imidazole, 2 mM $MgCl_2$, 2 mM KCl, 20 mM NaCl, 15 μM resazurin, 5 mM dithioerythritol, pH 6.8) and then suspended in the same buffer in 150-ml serum bottles at a final density of 20 to 25 mg of protein per ml (100% N_2 gas phase). The cells were freshly prepared for each experiment and could be stored on ice for 6 h without losing activity.

Experiments with cell suspensions. All assays were performed in 150-ml serum bottles containing 5 ml of assay mixture under strictly anaerobic conditions as described before (4). Samples from the gas phase (0.3 ml) were analyzed for CH_4 , CO_2 , and H_2 . Liquid samples (0.25 to 0.8 ml) were analyzed for pyruvate, acetate, amino acids, acetoin, acetolactate, and ATP. The assay mixture for the determination of the stoichiometry of pyruvate conversion contained 8 mM pyruvate, 60 mM BES, and 15 mg of cell protein per ml, and that for the determination of ATP synthesis and of the effects of arsenate and dicyclohexylcarbodiimide (DCCD) contained 60 mM pyruvate, 30 mM BES, 2.5 mg of cell protein per ml, and either 50 mM arsenate or 50 nmol of DCCD per mg of cell protein. Ethanolic stock solutions of DCCD were used. Generally the cells were preincubated for 10 min at 37°C in the presence of BES or DCCD before the addition of pyruvate.

Preparation of cell extracts. Crude cell extracts and ferredoxin-free cell extracts were prepared as described previously (4). For the determination of acetate kinase activity and phosphate acetyltransferase activity, crude cell extracts were desalted and depleted of cofactors by chromatography on Sephadex G-25 (22). The cell extract was stored at a concentration of about 10 mg/ml on ice for several hours or frozen at $-20^\circ C$.

Determination of enzyme activities. All enzyme assays were performed at 37°C. Stopped glass cuvettes, filled with 1 ml of assay mixture, were repeatedly evacuated and gassed with N_2 to achieve anaerobic conditions. One unit of enzyme activity is defined as 1 μmol of substrate consumed or product formed per min. Pyruvate:ferredoxin oxidoreductase activity was determined by monitoring the reduction of *C. pasteurianum* ferredoxin at 405 nm (ϵ_{405} [oxidized ferredoxin - reduced ferredoxin] = $14 \text{ mM}^{-1} \text{ cm}^{-1}$). The assay mixture contained 100 mM Tris-HCl (pH 8.1), 5 mM dithioerythritol, 0.07 mM ferredoxin, 0.1 mM CoA, 10 mM pyruvate, and 40 to 250 μg of extract protein.

Acetate kinase (EC 2.7.2.1.) activity was measured by monitoring the formation of ATP from acetylphosphate and ADP. The reaction was coupled with the reduction of $NADP^+$ via hexokinase and glucose-6-phosphate dehydrogenase. The assay mixture contained 100 mM Tris-HCl (pH 8.1), 5 mM dithioerythritol, 2 mM acetylphosphate, 5 mM $MgCl_2$, 10 mM glucose, 1 mM $NADP^+$, 2 mM ADP, 2.8 U of hexokinase, 1.4 U of glucose-6-phosphate dehydrogenase, and 3 to 12 μg of extract protein.

Phosphate acetyltransferase (EC 2.3.1.8.) activity was determined by monitoring the formation of acetyl-CoA from acetylphosphate and CoA at 233 nm (ϵ_{233} = $4.44 \text{ mM}^{-1} \text{ cm}^{-1}$). The assay mixture contained 100 mM Tris-HCl (pH 7.2), 1.7 mM glutathione, 30 mM NH_4Cl , 2 mM acetylphosphate, 0.3 mM CoA, and 6 to 12 μg of extract protein.

Analytical procedures. CH_4 , CO_2 , and H_2 were determined by gas chromatography (24). Acetate was determined enzymatically as described by Beutler (3).

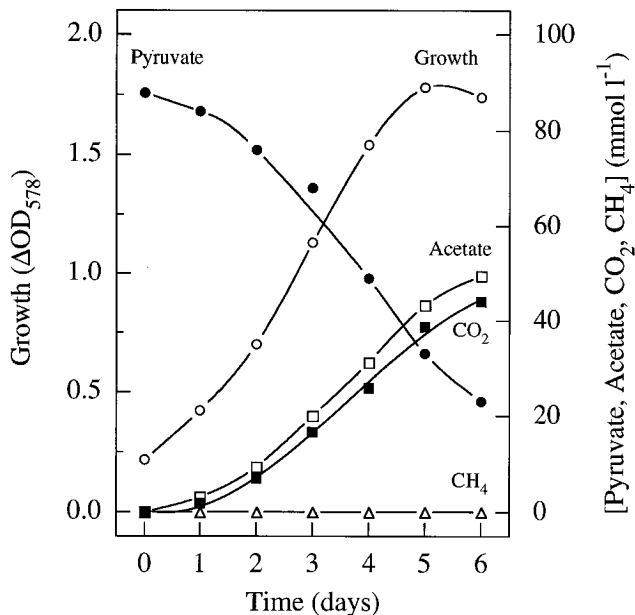


FIG. 1. Growth of *M. barkeri* (Fusaro) under nonmethanogenic conditions with pyruvate as the sole carbon and energy source in the presence of BES. Cells were grown at 37°C on a medium containing 88 mM pyruvate and 2 mM BES. *M. barkeri* grown on pyruvate in the absence of BES was used as the inoculum. A ΔOD_{578} of 1 corresponded to a cell concentration of 240 mg (dry weight) per liter. Consumption of pyruvate and formation of acetate, CO_2 , and CH_4 are given as millimoles consumed or formed per liter of culture.

Pyruvate was determined enzymatically with lactate dehydrogenase (17). Amino acids were determined by fluorescence high-pressure liquid chromatography as described by Algermissen et al. (1). The amino acid analyses were kindly performed by M. Nündel and E. Riedel (Freie Universität Berlin). The protein contents of cell extracts were determined by a modified Biuret method (5) with bovine serum albumin as the standard. Acetoin-butandiol was determined colorimetrically as described by Westerfeld (30). Acetolactate was determined as acetoin after decarboxylation with 3 M H_2SO_4 to acetoin and CO_2 (16).

The ATP content of the cells was determined by using the luciferin-luciferase assay as described by Kaesler and Schönheit (15). Samples (50 μl) of the cell suspension were transferred directly into 950 μl of ethanol (96%) at $-20^\circ C$ and rapidly mixed. Twenty microliters of the ethanol extract was added to 460 μl of buffer (100 mM Tris-acetate [pH 7.75], 2 mM EDTA). After addition of the luciferin-luciferase mixture (monitoring reagent) (Bio-Orbit, Turku, Finland), ATP was measured with a luminometer 1250 (Bio-Orbit).

RESULTS

Growth of *M. barkeri* on pyruvate in the presence of BES. *M. barkeri* previously grown by pyruvate conversion to CH_4 was transferred to a mineral salt medium containing pyruvate (87 mM) and 2 mM BES. The organism grew with a doubling time of about 30 h up to cell densities of about 400 mg (dry weight) of cells per liter. At the end of the exponential growth phase (5 days), 54 mM pyruvate was consumed, and acetate (44 mM) and CO_2 (39 mM) were detected as major fermentation products (Fig. 1). In addition, small amounts of reduced products such as alanine (0.7 mM), valine (0.7 mM), leucine (0.4 mM), isoleucine (0.3 mM), acetoin-butandiol (0.27 mM), and acetolactate (0.1 mM) were found. CH_4 was not detected. For each mole of pyruvate consumed in catabolism, about 0.9 mol of acetate and 0.8 mol of CO_2 were formed. The recovery of carbon and [H] from pyruvate in the fermentation products and cell mass were 93 and 88%, respectively, assuming that the approximate composition of the cell material of *M. barkeri* is equal to CH_2O . The amount of pyruvate required for cell carbon synthesis (5.5 mM pyruvate) was calculated according

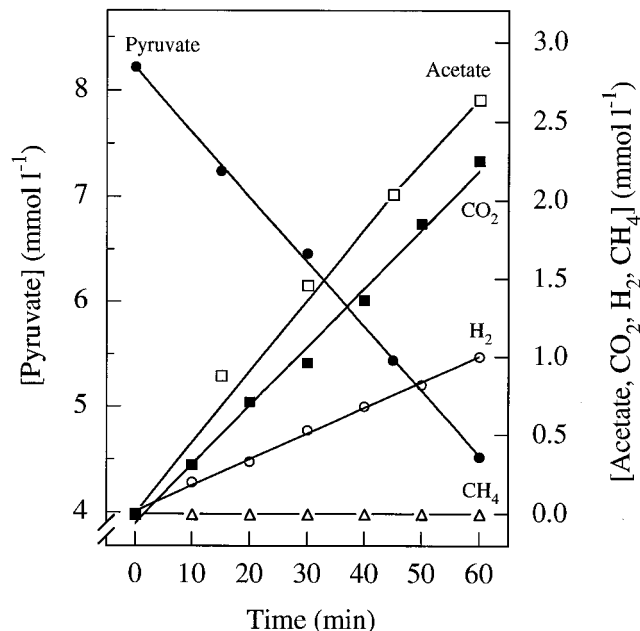


FIG. 2. Stoichiometry of fermentation of pyruvate to acetate, CO₂, and H₂ in cell suspensions of *M. barkeri* in the presence of BES. The assay was performed at 37°C in anaerobic imidazole-chloride buffer (pH 6.8). The assay mixture contained 15 mg of protein per ml and 60 mM BES. The gas phase was 100% N₂ at 150 kPa. The reaction was started by the addition of pyruvate (8.2 mM).

to the following equation: $2\text{CH}_3\text{COCOO}^- + 2\text{H}^+ + 2\text{H}_2\text{O} \rightarrow \text{CO}_2 + 5\text{CH}_2\text{O}$ (4). The molar growth yield (Y_{acetate}), determined at the end of the exponential growth phase, was 9 g (dry weight) of cells/mol of acetate formed.

Similar doubling times and molar growth yields were observed for six to eight generations on medium with pyruvate-BES. Upon further propagation, the doubling times increased and the cell yields decreased. Almost no growth was observed after 9 to 11 generations.

Enzyme activities. Cell extracts of *M. barkeri* grown on pyruvate-BES medium contained pyruvate:ferredoxin oxidoreductase (0.5 U/mg), phosphate acetyltransferase (12 U/mg), and acetate kinase (12 U/mg). Cell extracts of *M. barkeri* grown on pyruvate medium without BES contained pyruvate:ferredoxin oxidoreductase, phosphate acetyltransferase, and acetate kinase at specific activities of 0.8, 23, and 24 U/mg, respectively.

Cell suspension experiments. (i) Stoichiometry of pyruvate fermentation. Cell suspensions (15 mg of protein per ml) of *M. barkeri* previously grown on pyruvate were incubated in imidazole buffer (pH 6.8) containing 8.5 mM pyruvate and 60 mM BES. As shown in Fig. 2, the cells consumed pyruvate (3.7 mM) at a rate of about 4 nmol min⁻¹ mg⁻¹; about 0.8 mol each of acetate and CO₂ and 0.3 mol of H₂ per mol of pyruvate consumed were formed. In addition, small amounts (<0.04 mol/mol of pyruvate) of alanine, valine, lysine, acetolactate, and acetoin-butandiol were detected. CH₄ was not formed. The recoveries of carbon and [H] from the pyruvate fermentation were about 90 and 110%, respectively. ATP synthesis and the effects of arsenate and DCCD were measured at lower cell concentrations (2.5 to 5 mg of protein per ml) and at higher pyruvate concentrations (60 mM). Under these conditions, the rates of acetate, CO₂, and H₂ formation increased to 15 to 20 nmol min⁻¹ mg⁻¹. Acetate, CO₂, and H₂ were formed at approximate stoichiometries of 1:1:0.8 (see Fig. 4), indicating almost complete conversion of pyruvate to these products.

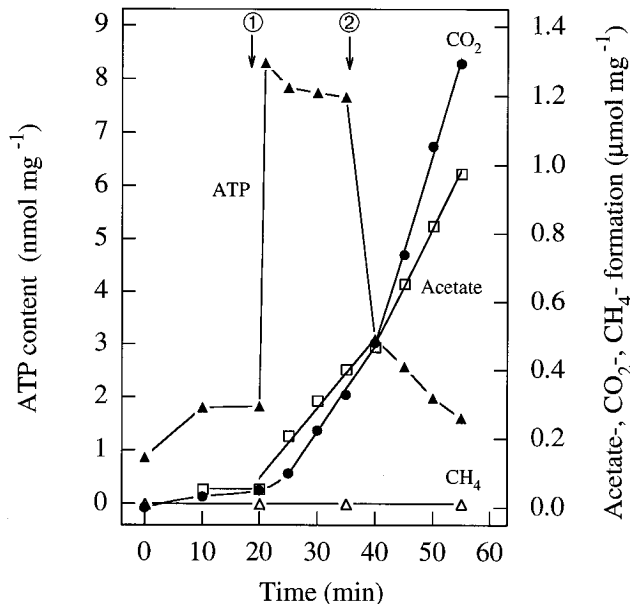


FIG. 3. Effect of arsenate on ATP synthesis under nonmethanogenic conditions coupled to conversion of pyruvate to acetate in cell suspensions of *M. barkeri*. The assay was performed at 37°C in anaerobic imidazole-chloride buffer (pH 6.8) containing 2.5 mg of protein per ml and 15 mM BES. The gas phase was 100% N₂ at 150 kPa. After 20 min (arrow 1), 60 mM pyruvate was added. After 35 min (arrow 2), 50 mM arsenate was added.

(ii) ATP synthesis coupled to fermentation of pyruvate to acetate and the effects of arsenate and DCCD. In the presence of 50 mM BES, cell suspensions (2.5 mg of protein per ml) of *M. barkeri* coupled the conversion of pyruvate to acetate, CO₂, and H₂ with the phosphorylation of ADP (Fig. 3, arrow 1). The cellular ATP content increased from 1.8 to 8.3 nmol/mg of protein upon addition of pyruvate. From the rates of ATP increase (6.5 nmol min⁻¹ mg⁻¹) (determined 1 min after pyruvate addition) and of acetate formation (20 nmol min⁻¹ mg⁻¹), a minimum stoichiometry of about 0.3 mol of ATP per mol of acetate was calculated (Fig. 3). In other, similar experiments, ATP/acetate stoichiometries of 0.3 to 0.5 were observed.

ATP synthesis in the complete absence of methanogenesis probably proceeds by the mechanism of substrate level phosphorylation. To test this hypothesis, the effects of arsenate, an uncoupler of ATP synthesis by substrate level phosphorylation, and of DCCD, an inhibitor of H⁺-translocating ATP synthase from *M. barkeri* (6, 12), were tested.

The formation of acetate, CO₂, and H₂ was stimulated by arsenate; a fourfold stimulation was observed at 50 mM arsenate (Fig. 4). At this concentration the rates of formation of acetate, CO₂, and H₂ were each about 60 nmol min⁻¹ mg of protein⁻¹. The effect of arsenate on the ATP content of *M. barkeri* is shown in Fig. 4. The addition of 50 mM arsenate (Fig. 3, arrow 2) resulted in a rapid decrease of the ATP content from about 8 nmol/mg of protein to the basal level (1 to 2 nmol/mg of protein). Concomitantly, the formation of acetate and CO₂ was stimulated.

Cell suspensions (5 mg of protein per ml) were incubated for 10 min either in the absence or in the presence of 50 nmol of DCCD per mg of protein before the addition of pyruvate (60 mM). In the absence of DCCD, the amounts of acetate, CO₂, and acetolactate formed per milligram of protein, determined 40 min after pyruvate addition, were 0.7, 0.68, and 0.07 μmol,

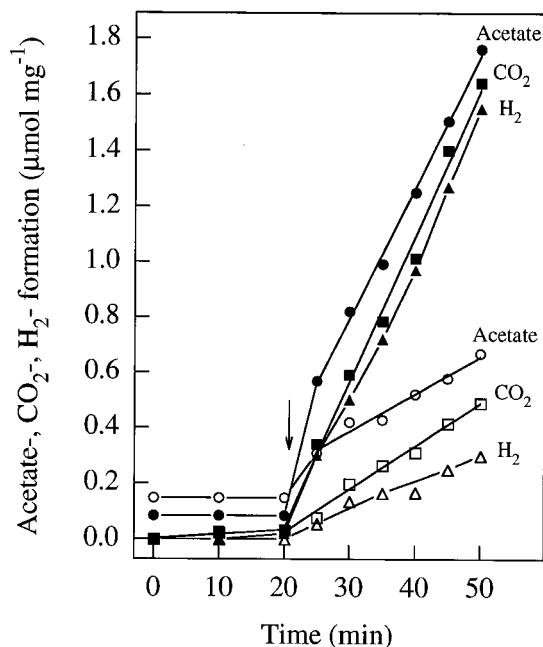


FIG. 4. Effect of arsenate on formation of acetate, CO_2 , and H_2 from pyruvate in cell suspensions of *M. barkeri*. The assays were performed at 37°C in anaerobic imidazole-chloride buffer (pH 6.8) containing 2.5 mg of protein per ml and 15 mM BES in either the absence (open symbols) or presence (closed symbols) of 50 mM arsenate. The gas phase was 100% N_2 at 150 kPa. The reaction was started by the addition of 60 mM pyruvate (arrow).

respectively. In the presence of DCCD, the amounts of acetate, CO_2 , and acetolactate formed per milligram of protein, determined 40 min after pyruvate addition, were 0.36, 0.8, and $1.6 \mu\text{mol}$, respectively. Thus, DCCD caused a shift of pyruvate fermentation from acetate to acetolactate. The reasons for this shift cannot be explained so far.

DCCD did not inhibit ATP synthesis coupled to acetate formation from pyruvate. The ATP content of the cells (5 mg of protein per ml) preincubated for 10 min in the presence of 50 nmol of DCCD per mg and 50 mM BES increased within 1 min from 2.5 to 9 nmol/mg upon the addition of pyruvate. From the rates of ATP increase ($6.5 \text{ nmol min}^{-1} \text{ mg}^{-1}$) (determined 1 min after pyruvate addition) and of acetate formation ($7.3 \text{ nmol min}^{-1} \text{ mg}^{-1}$), an apparent stoichiometry of 0.85 mol of ATP per mol of acetate was calculated. An ATP/acetate stoichiometry of near 1 is in accordance with ATP synthesis in the acetate kinase reaction by substrate level phosphorylation.

DISCUSSION

Growth of *M. barkeri* under nonmethanogenic conditions.

Upon complete inhibition of methanogenesis by BES, *M. barkeri* grew at the expense of pyruvate fermentation to acetate and CO_2 . ATP synthesis is coupled to acetate formation as the only energy-conserving site. Pyruvate conversion to acetolactate, acetoin-butandiol, alanine, and valine, which are formed during growth at low concentrations, is not coupled with energy conservation. Assuming a Y_{ATP} of 7 to 9 g (dry weight) of cells per mol of ATP with pyruvate as an anabolic substrate (7, 26), the molar growth yield, Y_{acetate} , of about 9 g (dry weight) of cells per mol of acetate formed indicated a stoichiometry of 1 mol of ATP per mol of acetate.

Growth of *M. barkeri* at the expense of pyruvate fermentation to acetate in the absence of methanogenesis was maintained for about 8 to 10 generations, after which growth ceased. The reason(s) for the limited capacity to grow by pyruvate fermentation to acetate cannot yet be explained. The specific activities of the catabolic enzymes, pyruvate:ferredoxin oxidoreductase, phosphate acetyltransferase, and acetate kinase (see below), do not change significantly.

Mechanism of ATP synthesis coupled to fermentation of pyruvate to acetate. The proposed pathway of pyruvate fermentation to acetate is shown in Fig. 5. After transport into the cell, probably via passive diffusion (4), pyruvate is oxidized to

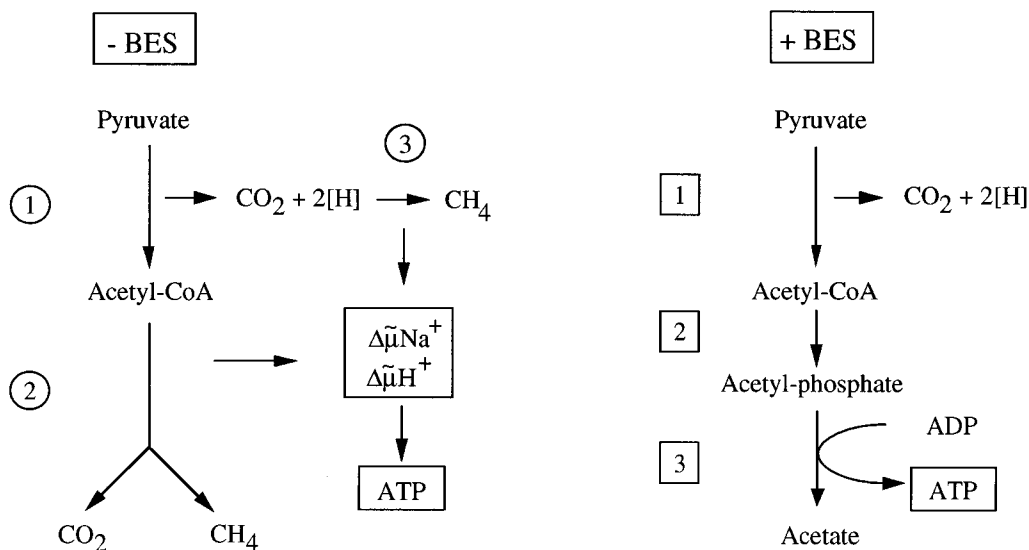


FIG. 5. Proposed pathways of pyruvate catabolism and mechanisms of ATP synthesis during growth of *M. barkeri* under methanogenic conditions (in the absence of BES [-BES]) and under nonmethanogenic conditions (in the presence of BES [+BES]). For the pathway without BES, 1 indicates pyruvate:ferredoxin oxidoreductase, 2 indicates enzymes of acetyl-CoA conversion to CH_4 and CO_2 (10, 28), and 3 indicates enzymes of CO_2 reduction to CH_4 (8, 27); ATP is formed by electron transport phosphorylation (19, 23). For the pathway with BES, 1 indicates pyruvate:ferredoxin oxidoreductase; 2 indicates phosphate acetyltransferase, and 3 indicates acetate kinase; ATP is formed by substrate level phosphorylation.

acetyl-CoA, CO₂, and reducing equivalents via pyruvate:ferredoxin oxidoreductase; acetyl-CoA is then converted to acetate via acetylphosphate via reactions involving phosphate acetyltransferase and acetate kinase. All three enzymes are present at high specific activities in cells grown on pyruvate in the presence of BES. Reducing equivalents generated by pyruvate:ferredoxin oxidoreductase are reoxidized during formation of butandiol or amino acids derived from pyruvate. In contrast to growing cultures, cell suspensions liberate significant amounts of reducing equivalents as molecular hydrogen. In accordance with this, pyruvate-grown cells contain high levels of hydrogenase activity (4).

Y_{acetate} values and the results of enzyme studies and cell suspension experiments support the existence of this pathway and indicate that ATP is formed during pyruvate fermentation in the acetate kinase reaction by substrate level phosphorylation, as follows. (i) From the Y_{acetate} values, the stoichiometry of moles of ATP to moles of acetate was close to 1, as predicted from the pathway. (ii) In cell suspensions, ATP/acetate stoichiometries of about 0.5 (up to 0.9 in the presence of DCCD) were measured directly. (iii) Arsenate exerts a "classical" uncoupler effect in cell suspensions; it inhibited ATP synthesis and concomitantly stimulated acetate formation. Stimulation factors of about 4 indicate a tight coupling between ATP synthesis and acetate formation. (iv) DCCD, an inhibitor of H⁺-translocating ATP synthase and thus of electron transport phosphorylation, did not inhibit ATP formation coupled to acetate formation. However, DCCD, used at concentrations similar to those used in this study (30 to 50 nmol/mg of protein), has been found to completely inhibit ATP synthesis coupled to methane formation, e.g., from H₂-CH₃OH, in cell suspensions of *M. barkeri* (6), which is in accordance with ATP formation by the mechanism of electron transport phosphorylation.

Cell suspensions of *M. barkeri* have been reported to catalyze the formation of acetate from methyl iodide, CO₂, and H₂ (17). Enzyme studies and the effects of arsenate and DCCD on ATP synthesis and acetate formation have been interpreted to indicate that acetate is formed from acetyl-CoA via acetylphosphate and that ATP is generated in the acetate kinase reaction by the mechanism of substrate level phosphorylation. However, growth of *M. barkeri* at the expense of acetate formation from methyl iodide, CO₂, and H₂ has not been demonstrated.

M. barkeri MS and *Methanococcus* species have also been reported to utilize pyruvate in cell suspensions. *M. barkeri* converts pyruvate mainly to acetolactate (up to about 0.4 mol/mol); in addition, small amounts of valine, acetate, and methane were formed (21). *Methanococcus* species convert pyruvate to acetate and methane (31).

The data reported here and by Bock et al. (4) indicate that *M. barkeri* is able to grow on pyruvate by two different catabolic pathways and to generate ATP by two different mechanisms. The two proposed pathways are shown in Fig. 5. In the absence of BES, pyruvate is converted to CH₄ and CO₂. CO₂ is reduced to CH₄ by reducing equivalents (2[H]), and acetyl-CoA is converted to CO₂ and CH₄. These partial reactions are apparently coupled with the generation of transmembrane electrochemical H⁺ and Na⁺ gradients ($\Delta\mu_{\text{H}^+}$ and $\Delta\mu_{\text{Na}^+}$), in analogy to the proposed coupling sites involved in conversion of acetate to CH₄ and CO₂ and in reduction of CO₂ to CH₄. ATP is then synthesized by the mechanism of electron transport phosphorylation via H⁺-translocating ATP synthase (8, 19, 23, 27). In the presence of BES, methanogenesis is completely inhibited and pyruvate is converted via acetyl-CoA to acetate. ATP is formed by the mechanism of substrate level phosphorylation in the acetate kinase reaction.

The observation of growth of *M. barkeri* due to pyruvate fermentation to acetate is the first observation of growth of a methanogen at the expense of an energy metabolism different from methanogenesis. This facultative growth of *M. barkeri* is a prerequisite for the generation and analysis of mutants defective in enzymes of various methanogenic pathways. Such mutations are lethal if methanogenesis is the only mode of energy metabolism.

The growth of *M. barkeri* by conversion of pyruvate to methane (4) and to acetate (this paper) requires high concentrations (>20 mM) of pyruvate, probably because the lack of a high-affinity uptake system (for a discussion, see reference 4). Since pyruvate is not present as a free fermentation product in ecosystems, it is not a natural substrate for methanogens. However, as an artificial substrate, pyruvate provides a useful tool for the investigation of various aspects of energy metabolism in acetoclastic methanogens.

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