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Use of Anaerobic Bacteria in Generating Fuels and Chemicals from Coal

Scientific Note

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INTRODUCTION

The breakthrough in the utilization of coal will come by increasing its share in the total energy market. Presently, coal is not being utilized because other fossil energy resources are more economical and burn more environmentally safely. The combustion of coal to produce coal-gas or syngas is fairly well known. It appears that biological processes for the utilization of coal products, especially syngas, may provide a cost effective means to obtain alternative industrially important chemicals.

The use of microorganisms for the solubilization of coal is being attempted at various laboratories (*1–3*). However, little attention has been given to the utilization of syngas by microbial fermentation (*4*).

Clostridium thermoaceticum (*5*) in glucose-limited cultures grows at the expense of CO and CO_2 and autotrophically utilizes syngas. The hydrogenase of this organism functions in both the production and consumption of H_2 and both heterotrophic and autotrophic roles exist for this enzyme (*6*) in the production of acetate. We have been looking into the ability of *C. thermoaceticum* to utilize syngas (a mixture of CO, CO_2, and H_2) and to see if this bioconversion can yield useful fuels and/or chemicals. This organism also contains the enzymes CO-dehydrogenase and formate dehydrogenase, which are both required for the bioconversion of syngas and the production of acetic acid. In an effort to engineer *C.*

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thermoaceticum to overcome its natural physiological limitation as a syngas utilizer, we decided to look for naturally occurring plasmids and phages that may be developed as cloning vectors.

We shall give some data on the basic physiology and genetics of *C. thermoaceticum*, a potential syngas or coal-gas utilizer.

MATERIALS AND METHODS

Strains

C. thermoaceticum (originally isolated by Fontaine in 1942) was obtained from H. G. Wood, Case Western Reserve University, Cleveland, OH. *E. coli* K-12 harboring the plasmid pJMB8:Tn5(11 kb) was received from D. Berg, Washington University, St. Louis.

Media

The organism was grown at 55°C in crimp-sealed bottles (Wheaton serum, 150 mL) containing 40 mL of glucose medium under 100% CO_2 (*7*). This has been called normal or undefined (UM). The defined (DM) and the minimally defined medium (MDM) were also used. In DM, tryptone and yeast extract are replaced with amino acids and vitamins. In MDM, the solution C was devoid of even all the vitamins and amino acids except 2 mg nicotinic acid/L of the medium. Cells were transferred into fresh media anaerobically every 48–60 h. *E. coli* was grown in Luria broth, supplemented with the antibiotics tetracycline and ampicillin.

Plasmid Screening

Bacteria harvested from 40 mL cultures of *C. thermoaceticum* in three types of media (UM, DM, MDM) were washed with 7 mL TES buffer (50 m*M* Tris, 3 m*M* NaCl, pH 8, with HCl). Pellets were resuspended in 1 mL of 50 m*M* Tris-HCl, pH 8, and kept on ice for 15 min; 0.5 mL lysozyme (10 mg/mL) in 25 m*M* Tris-HCl, pH 8, was then added. Cells were well vortexed and kept on ice for 30 min; 0.5 mL EDTA (Na_2), pH 8, was added; followed (after 15 min on ice) with 1 mL of 2.5% SDS in 50 m*M* Tris-HCl, pH 8, and 25 m*M* Na_2 EDTA. The mixture was kept on ice for 30 min when 1 mL of 5 *M* NaCl was added and lysate was stored at 4°C for 30 min. The supernatant was heated to 60°C for 20 min to inactivate the nucleases. This liquid was then treated with RNAse (20 mg/mL, final volume). One mL of the supernatants, after the above treatments, was transferred into several Eppendorf tubes (1.5 mL) and 100 μL of 0.1 *M* NaAC, 1*M* Tris-HCl, pH 8, was added to the tubes. After thorough mixing, 0.4 mL of cold (−20°C) ethyl alcohol was added to the tubes, which were then incubated at −70°C (deep freezer) for 30–60 min. The supernatant was removed by spinning in a microcentrifuge for 5 min. The DNA precipitate was washed two or three times with ethyl alcohol

and desiccated dry under vacuum for 30 min and resuspended in 0.0005 mL TES buffer, then stored at 4°C. The plasmid DNA from *E. coli* K-12 was extracted by the method described by Birnboim and Doly (*8*).

Purification of Plasmid DNA

The bacteria harvested from a 5-L culture were subjected to DNA extraction by ethanol precipitation as described above (volumes of buffer, etc., were adjusted accordingly). The DNA so obtained was further purified by cesium chloride, buoyant-density gradient centrifugation (*9*), and the supercoiled plasmid DNA band was collected by puncturing the tube under UV light. Ethidium bromide was removed using isopropanol (*10*). The DNA was stored for further experiments.

Transformation of Competent Cells

Preparation and transformation of competent cells for *E. coli* and *C. thermoaceticum* was done by treating the cells with $CaCl_2$ and by heat shock. The experiments were conducted inside a Coy anaerobic chamber to avoid oxygen toxicity to *C. thermoaceticum* cells. The transfers of transformed cells into growth media were done under a CO_2 atmosphere. Plasmid DNA (pJMB8:Tn5) from *E. coli* was used to transform cells of *C. thermoaceticum* and the plasmid from *C. thermoaceticum* (30 kb) was used to transform *E. coli*.

Gel Electrophoresis

Horizontal, submerged agarose (0.9 to 1.0%) gels were run at 50 V (60 mA) for 3–6 h. The bands were visualized, after staining the gels with ethidium bromide (1.5 μg/mL) through the long-wavelength UV transilluminator. The tray buffer used was TES (40 m*M* Tris-HCl, pH 7.6, 20 m*M* sodium acetate, and 2 m*M* EDTA).

Curing of Plasmids

Cells grown in DM and MDM and showing plasmid bands for *C. thermoaceticum* were treated with acridine orange. The acridine orange was added at varying concentrations into the medium, and the cells were subsequently transferred several times in media containing acridine orange (*11*), and were analyzed by gel electrophoresis for the presence or absence of plasmids.

Assay for Enzymes

The cells that contained naturally occurring plasmids and those that were cured of plasmids by treating them with acridine orange, were used separately to assay for the presence of enzymes, especially hydrogenases. Cell extracts were prepared by carrying out all the procedures in-

side the Coy anaerobic chamber (except centrifugation in sealed tubes) and were stored in 2 mL-crimp-sealed bottles at 4°C. Estimation of the total proteins in the cell extracts and estimation of specific enzymes was done as described by Ljungdahl and Anderson (*12*).

RESULTS AND DISCUSSION

We have obtained evidence for the presence of a naturally occurring plasmid (so far unknown) in *C. thermoaceticum*. Several other species of *Clostridium* are also known to harbor plasmid (*13*). The agarose gels show a clear, sharp band for a 30-kb size plasmid (Fig. 1). The smaller *E. coli* plasmid, pJMB8 (with the transposon Tn5) of 11-kb size, migrates at different rate due to its smaller size. Plasmid DNA was purified by CsCl density gradient and the two separate bands for chromosomal (upper)

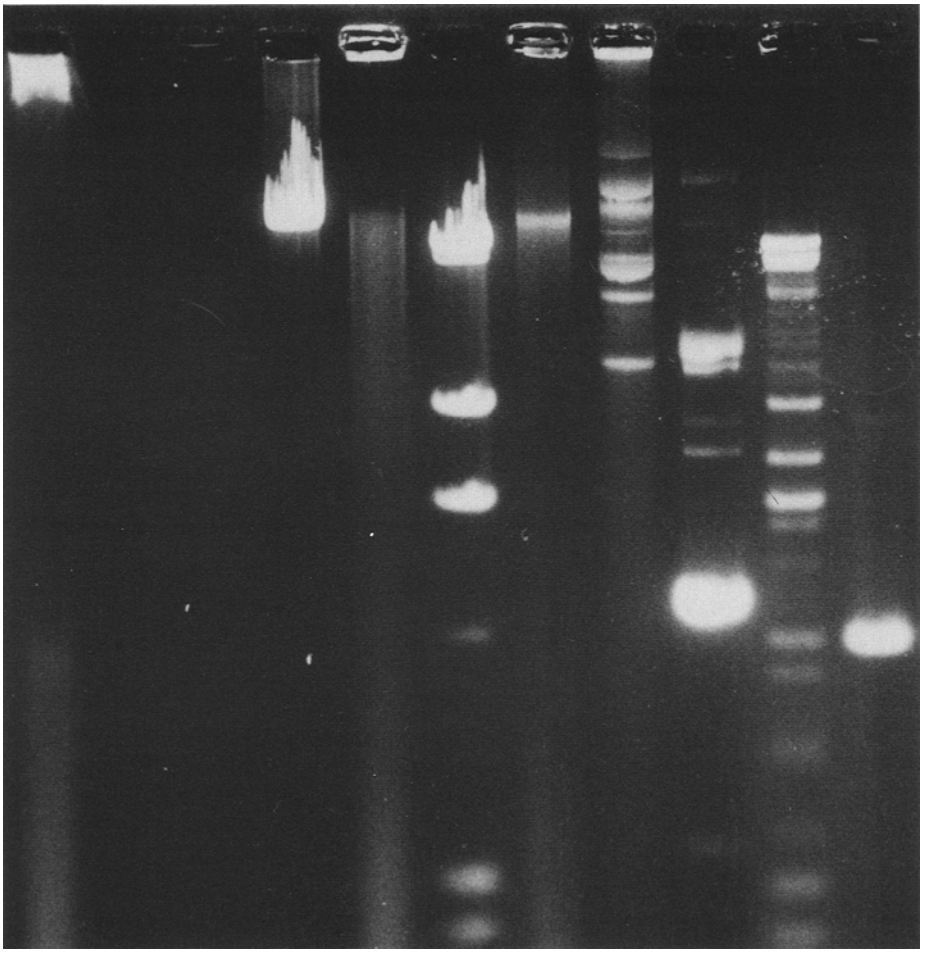

Fig. 1. Plasmid profiles on agarose gel.

and the plasmid (lower) DNA was further evidence to this fact (Fig. 1). The disappearance of the plasmid band on agarose gels from cells treated with acridine orange, indicates the plasmid nature of this DNA. The plasmid DNA that we obtained from *C. thermoaceticum* has been subjected to digestion by restriction enzymes (especially *Bam* H1, *Eco*R1, *Sal* 1, and *Hind* III) and a restriction map is being developed. Since the genetics of anaerobes is still little explored (due to the lack of proper techniques to handle them, especially in generating mutants, accomplishing replica plating on solid media, etc.), we think this plasmid can give us some insight into basic physiological mechanisms if we transfer the plasmid DNA into a well-established system like *E. coli* (*14*). Attempts to transfer the plasmid DNA from *C. thermoaceticum* to *E. coli* cells was successful since we noted an extra band in transformed *E. coli* cells that comigrate with *C. thermoaceticum* plasmids. *C. thermocellum* and *C. butyricum* genes have already been cloned in *E. coli* and their expression has also been confirmed (*15*). We are trying to perform further experiments with transformed *E. coli* cells that harbor the *C. thermoaceticum* plasmid. The back transformation of *C. thermoaceticum* plasmid DNA from *E. coli* did not work, and we feel that this failure was due to technique difficulties. It may thus be said that it is easy to transfer a thermophilic gene into a mesophilic environment, while it is relatively difficult to achieve the transfer the other way.

Hydrogenase enzyme activity is associated with a plasmid in *Alcaligenes eutrophus*. Our results of enzyme assays in plasmid-cured *C. thermoaceticum* strains showed no significant reduction in the specific activities of CO dehydrogenase, formate dehydrogenase, and hydrogenase due to the loss of the plasmids in the cells (Table 1). Hence, it can be concluded that the plasmid that we have detected is not associated with these key enzymes of acetate biosynthesis. But the identification of plasmids in *C. thermoaceticum* and its further characterization (which is underway) should go a long way toward developing a cloning vector for gene transfer in industrially important thermophilic and anaerobic acetogens and methanogens.

Table 1
Enzyme Levels in Normal (Control) and AO (Acridine Orange)-treated, Plasmid-cured Cells of *C. Thermoaceticum*

	Enzymes (specific activity)		
Cells	Carbon monoxide dehydrogenase	Hydrogenase	Formate dehydrogenase
Control	20.5	7.1	5.1
AO-treated	19.3	7.2	5.4

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