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MICROBIAL ECOLOGY OF AN ANIMAL WASTE-FUELED  
INDUCED BLANKET REACTOR

by

Steven C. Curtis

A thesis submitted in partial fulfillment  
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY  
Logan, Utah

2006

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## ABSTRACT

Microbial Ecology of an Animal Waste-Fueled  
Induced Blanket Reactor

by

Steven C. Curtis, Master of Science

Utah State University, 2006

Major Professor: Dr. Jeffery R. Broadbent  
Department: Nutrition and Food Sciences

Use of an induced blanket reactor (IBR) to break down organic matter into methane is a financially attractive method to reduce the environmental impact of animal or industrial waste. In order to better understand the biological processes involved with the conversion of waste to biogas by an IBR, it is necessary to gain a better understanding of the microorganisms and their roles in the reactor. Molecular techniques based on the isolation of 16S rDNA were used in order to avoid the limitations posed by conventional culture-based techniques. Total DNA was extracted and amplified using universal primers specific to eubacteria and archaea with the purpose of identifying the dominant microorganisms in the IBR. The amplified DNA was separated based on its sequence composition by denaturing gradient gel electrophoresis (DGGE). Several bands were then excised, cloned, and sequenced, in order to characterize the phylogenetic affiliation of many of the microorganisms and create a useful molecular fingerprint. By using this approach, close relatives of several microorganisms that are typical in anaerobic digestion have been

identified, including species of *Clostridium*, *Flavobacterium*, *Bacteroides*, *Spirochaeta*, *Methanobrevibacter*, and *Methanosarcina*. Several species were also identified whose role in the reactor is not completely understood, consisting of relatives of *Dehalococcoides*, *Planctomyces*, *Aequorivita*, and *Sedimentibacter* species. The information obtained in this project may enable refinements that promote desirable reactions and enhance reactor efficiency.

(58 pages)

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## INTRODUCTION

From 1978 to 1992 the population of agricultural animals in the United States increased by 4.5 million (approximately 3%), but the total animal feeding sites decreased (19). The consolidation of these animals creates a variety of problems for adjacent communities, including oxygen depletion and disease transmission in surface water, pathogen and nutrient contamination in surface and ground water, methane and ammonia air emissions, and excessive buildup of toxins, metals, and nutrients in the soil (19, 20, 26, 34, 39, 52). These are common problems associated with breweries, slaughterhouses, dairy farms, and other agricultural and consumer industries that produce large amounts of organic waste.

The concentration of organic wastes and the associated environmental problems are facing more stringent review by the public, government, and industry; creating the need for innovation. One available solution is the use of an anaerobic reactor to break down the undigested plant matter and other available nutrients into more compact and environmentally benign products (20, 34). Anaerobic digestion also offers the benefit of providing an additional source of energy through the collection of methane produced in the reactor (4, 34). While the microbiota of an anaerobic reactor may include many organisms found in the rumen, the microbiology of the two systems is, generally, very different (4, 36, 38). The rumen and anaerobic reactors are both considered stable because they produce similar reactions, qualitatively and quantitatively, over long periods of time (60). There are however, short term variations in the microbial populations and end-products during these steady-states (25, 60).

The rumen is a large pre-gastric fermentation chamber that sustains a rich community of microorganisms, including anaerobic and facultative anaerobic eubacteria, archaea, fungi,

and ciliates, which rapidly colonize and digest feed particles (26, 88). Carbohydrate polymers in plants are indigestible to most animals, but can be hydrolyzed and fermented by a range of microorganisms in the rumen (46). However, the fiber digestion in the rumen is not optimal; this is supported by the fact that 20-70% of the fiber recovered from feces is still fermentable (36, 46, 88).

An induced blanket reactor (IBR) is similar to an upflow sludge blanket anaerobic bioreactor in that both are designed to build and maintain an active microbial population to break down available carbohydrates and polymers as they are pumped through the chamber (7, 34, 35). An IBR has an influent waste port in the bottom of the reactor where the sludge blanket forms; and this area is thick with solids (34, 35). Since anaerobic digestion is generally very time-consuming due to the slow growth of hydrolytic bacteria, the IBR is designed to accelerate this process through the retention of the majority of the active microbiota in the sludge layer (34, 35). The microbial population in the reactor reduces the percent solids, pathogen concentration, and the chemical oxygen demand of the animal waste by fermenting the carbohydrates that are present and producing, among other things, hydrogen and methane gas as by-products (4, 20, 34, 35, 52, 60, 78). The methane is captured and burned to produce electricity.

While the microorganisms that inhabit and contribute to IBR functions have not been investigated to date, many of the more common microorganisms associated with anaerobic fermentation are known. Facultative anaerobes, such as *Escherichia coli* and various lactic acid bacteria, may be responsible for reducing the digester contents, by absorbing the oxygen that is present in the influent and fermenting many of the readily available carbohydrates (36, 89). Hydrolytic, cellulolytic, and proteolytic microorganisms break down the complex

carbohydrates and proteins found in plant matter, releasing free carbohydrates and other by-products into the environment (45, 88). Several species of eubacteria known for the cellulolytic and proteolytic activities may contribute to these processes, including *Citrobacter freundii*, *Ruminococcus flavefaciens* and *R. albus*, as well as various species of *Clostridium*, *Lactobacillus*, *Enterobacteriaceae*, *Prevotella*, and *Bacteroides* (4, 12, 36, 38, 46, 60, 66, 74, 88, 93). As the complex plant polysaccharides are digested several species of anaerobic fermenters break mono- and disaccharides into volatile organic acids, this group is made up of species of *Bacteroides*, *Flavobacterium*, *Spirochaeta*, *Acetobacterium*, *Eubacterium*, *Clostridium*, and *Desulfobacter*.

In addition to eubacteria, anaerobic fungi, such as *Neocallimastix*, *Piromyces*, *Orpinomyces*, and *Anaeromyces* species, colonize plant tissue in the rumen and are active in fiber degradation (2, 46). The anaerobic fungi have the ability to physically rupture the outer plant wall due to the penetrative nature of the rhizoids or hyphae and produce high concentrations of cellulases and xylanases and other enzymes active against "crystalline" cellulose (highly ordered because of hydrogen bonding) (2, 46, 66).

The rumen also contains ciliates that contribute a small percentage of the cellulase activity in the rumen (88, 89, 93). Ciliates are also helpful in maintaining the pH of the rumen, and increasing the concentration of available nitrogen (88, 89).

Finally, methanogens convert the released volatile organic acids as well as free hydrogen and carbon dioxide into methane (4). The organisms that may be encountered in this group are *Methanobacterium*, *Methanobrevibacter*, *Methanococcus*, and *Methanosarcina* species (4, 36, 71, 74, 79, 92).

Anaerobic digestion is a unique and complex process with established economic advantages. Increased use of anaerobic digestion is limited by the extraordinary amount of failures that are associated with the reactors, including mechanical and biological problems (4, 34). A common biological problem in reactors is a drop in pH, and as methanogens are primarily responsible for the removal of acids; methanogens and their interactions is an area where attention should be placed (4, 36). By gaining a more fundamental understanding of its microbiological constituents, the process can likely be made even more efficient. Among other things, research is needed to determine if any part or parts of the complex process are limiting and thus are amenable to control measures that could improve methane production (4, 7, 46, 60).

The purpose of this project is to characterize the microbial ecology of an active IBR. The microbiota of the bioreactor will be identified through the use of a non-culturing technique called denaturing gradient gel electrophoresis (DGGE). It is our hypothesis that identification of microbes that predominate in an IBR will provide new insights on the metabolic processes that drives the reactor and present new strategies to improve reactor efficiency.

## LITERATURE REVIEW

### Anaerobic Biodegradation

Anaerobic waste digestion is a process that breaks down residual organic matter into a gaseous mixture composed primarily of methane and carbon dioxide through the concerted metabolism of a diverse microbial community (36, 50). In addition to the obvious benefit of harvesting biogas, anaerobic digestion has the advantage of a more environmentally-friendly residual waste (19). Despite the key role of microbes in this process, basic understanding of the microbial ecology of the system has been very limited (36).

Microbial ecology is a term used to describe the interactions among microorganisms and between microorganisms and their environment (57). In order to better understand microbial ecology, a fundamental understanding of diversity, distribution, and function of the microorganisms must be obtained (40, 65).

The microbial ecology of an anaerobic reactor has been broken down into three steps, hydrolytic, acidogenic, and methanogenic (36, 38, 50). While the microbial composition of the first two steps may be controlled by the composition of the influent, the methanogenic population is largely controlled by the oxidation-reduction status of the sludge (22, 81). It appears that the limiting step of anaerobic digestion may not be restricted to one metabolic process, but instead varies due to environmental factors such as, hydraulic loading rate, temperature, and the wastewater characteristics (50, 68, 77).

Animal waste-fueled anaerobic digesters are subject to continuous inoculation with rumen microbiota along with air- and soil-borne microorganisms, and it is likely that most species could be present in such a digester, including aerobes (36). As they pass through the

digester, aerobes and some facultative anaerobes will likely be displaced by anaerobes and eventually die off. The decline of these organisms, which include many species of human pathogens, is one of the benefits of a digester (20, 39).

The hydrolytic step consists of cellulolytic and proteolytic microorganisms that are responsible for the breakdown of undissolved carbohydrate polymers and proteins into soluble intermediates (36, 50). About 50-90% of the eubacteria in this step bind to the plant cell wall in order to avoid ciliate predation and to prevent the premature degradation of their metabolic enzymes by free proteases in their ecosystem (93). The organisms attach to the cellulose fibers through the use of a cellulosome (an extracellular, multienzyme complex produced by the bacteria that aid in attachment and degradation of cellulose) (46, 88, 93).

The second, acidogenic, step, occurs when simple carbohydrates are fermented into volatile fatty acids like acetic, propionic, and butyric acid (50, 68). This step also includes facultative anaerobes that are capable of absorbing any oxygen present in the digester, thus aiding growth of anaerobes (22, 36, 38). This step includes hydrogenogenic species that convert propionic and butyric acids into acetic acid and hydrogen (50). The pH of the reactor is usually maintained near neutrality by the microbial production of ammonia which is especially important because the organisms in the methanogenic step are not acid-tolerant (50).

The final step is the methanogenic step. The only species capable of methanogenesis are a unique group of archaea (5). The methanogenic species do not catabolize the original organic products directly, but use only the fermentation end products produced in the acidogenic step. Methane is produced from acetate or  $H_2$  and  $CO_2$  by methanogens such as assorted species of *Methanobrevibacter*, *Methanothermobacter*, *Methanosarcina*, etc. (15,



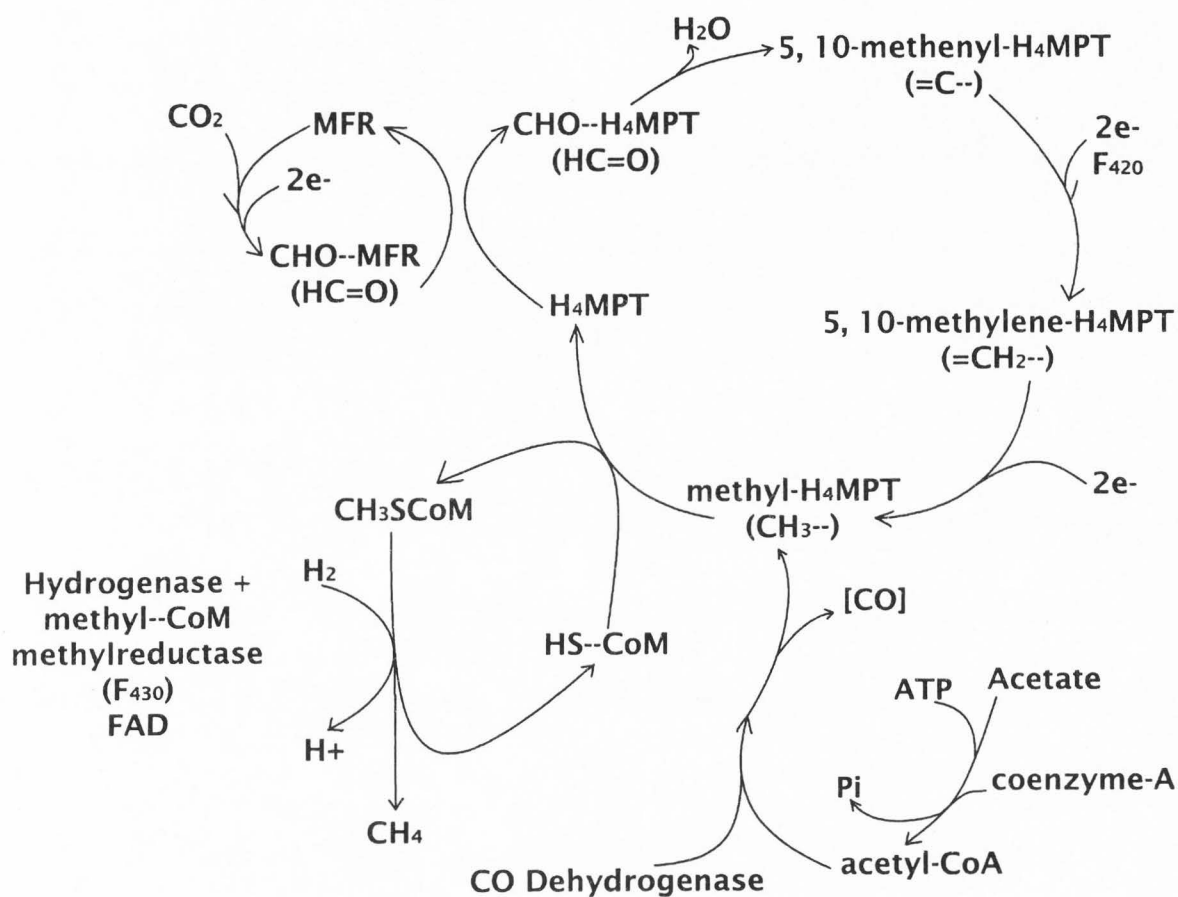


FIG. 1. Pathway for CH<sub>4</sub> synthesis from CO<sub>2</sub> and acetate in methanogens<sup>1</sup> (5, 69).  
<sup>1</sup>Cofactor abbreviations: methanopterin (MPT), methanofuran (MFR), and 2-mercaptoethanesulfonic acid or coenzyme M (CoM). The nature of carbon intermediates is indicated in parentheses.

22). Methanogens produce methane via a pathway that involves several unusual coenzymes (see Fig. 1) (5, 69). In the first step, Carbon Dioxide is bound to methanofuran (MFR) at the formyl reduction level and is further reduced to the methenyl, methylene, methyl, and finally methane levels while successively bound to the coenzymes tetrahydromethanopterin (MPT), 2-methylthioethanesulfonic acid, and 2-mercaptoethanesulfonic acid (CoM) (5, 69). The electrons from  $H_2$  are passed sequentially through coenzyme  $F_{420}$  and methanophenazine to heterodisulfide reductase creating a proton motive force. Energy is then created in the last step of methanogenesis with the reduction of the CoB-CoM complex by the enzyme heterodisulfide reductase (5).

While more energetically favorable, the acetate is a bit more involved. The addition of coenzyme-A to acetate, forming acetyl-CoA, is the first step in the process. This reacts with carbon monoxide dehydrogenase and the methyl group of acetyl-CoA is donated to a corrinoid-containing protein. The methyl group is then donated to CoM, forming  $CH_3$ -CoM, and the reaction proceeds as described for  $CO_2$  (5). Because some methanogens can use various fermentative end products of other species, they tend to aggregate and form syntrophic associations with species that provide the necessary substrate for methane formation (5, 15, 83).

Initially, research into the microbial ecology of anaerobic digestion was done using various culture-dependent techniques to identify and characterize the microorganisms in the reactor (36, 88). While useful for determining the presence of certain microorganisms, culture-dependent methods are limited by the fact that relatively few organisms are able to grow on the laboratory media used (9, 28, 71). The culture bias is explained in part by our lack of understanding of the necessary conditions for growth of some organisms, their

interdependency on other organisms for survival, or both (3, 28, 55, 57, 75, 91). It is now well recognized that only a small fraction, from one to twenty percent, of all microorganisms can be cultured (3, 28, 55, 57, 75, 91). Because of this, studies of microbial ecology have moved away from culture based techniques to molecular identification methods (27).

### **Importance of Non-Culture Techniques**

DNA-based methods allow for the identification of microorganisms, while diminishing the bias created by traditional culturing techniques (36, 52, 70, 71, 78, 86, 87, 91). A technique developed in 1983 made it possible to detect and clone rare sequences of DNA. Polymerase chain reaction (PCR) relies on the amplification of target DNA sequences by a DNA polymerase. Two specific oligonucleotides are synthesized to hybridize with the target strand of the DNA; one upstream to the 5' end of the target and a downstream primer that is complementary to the opposite strand. The 3' end of these oligonucleotides serve as primers for the initiation of replication of the original strands (51). The *Taq* polymerase used in this replication was isolated from a thermophilic bacterium, *Thermus aquaticus* (51). This heat-stable polymerase is added to the PCR reaction along with the oligonucleotide primers, template DNA, and free DNA base pairs. The reaction mix is heated to 94° C to denature the target DNA, and then cooled to the annealing temperature of the primers, which hybridize to the template DNA. The PCR mix is then heated to 72° C for the *Taq* polymerase to begin attaching the free DNA base pairs to the 3' end of the primers (51). This process is then repeated so that the fragment between the base pairs is exponentially amplified.

Most molecular techniques rely on the amplification of 16S rDNA because it is a highly conserved gene, is found in all eubacteria and archaea, and constitutes a relatively large

fraction of microbial biomass (3, 16, 69). The degree of conservation of 16S rDNA likely results because of its critical role in cell function, as opposed to genes needed to make enzymes (16). Mutations in the genes of enzymes can usually be tolerated more frequently since they may affect structures not as unique and essential as RNA (a bacterium with a mutation in a lactose gene may use other metabolites) (16). The structure of 16S rDNA changes very slowly with time, likely because of its important role in transcription. The 16S rDNA is about 1,550 base pairs long and codes for ribosomal RNA. The 16S rDNA is transcribed to RNA and constitutes part of the smaller 30S subunit (8). 16S rDNA is so highly conserved because the transcribed RNA folds into a defined structure with many short duplex regions, and a mutation in the gene would affect the secondary and tertiary structure of the gene (8).

Because the rDNA contains variable and stable sequences, both closely related and distantly related microorganism can be compared (69). The sequencing of 16S rDNA is an established tool for phylogenetic analysis of microorganisms in any environment. Molecular techniques used with environmental samples may be broken down into three general steps: DNA extraction, amplification, and sequencing. Each of these steps has a unique set of challenges (1, 55, 61).

One of the challenges associated with DNA extraction from environmental samples is the co-purification of humic acids with the DNA (52, 97, 104), because humic acids interfere with PCR amplification by inhibiting the DNA polymerase (47, 54, 97). Cell extraction techniques are not prone to contamination by humic acids, but do not have as high of DNA yield. They can also produce a more biased community sample than direct lysis of a total environmental sample, the most commonly used method (55). In order to remove the

contaminating humic acids, DNA binding columns or filters have been combined with direct lysis (55).

16S rDNA fragments in the extracted DNA are amplified and collected by PCR with universal primers. Universal primers are designed to hybridize to a conserved region of the 16S rDNA of a broad group of microorganisms. While universal primers are important for the amplification of 16S rDNA from a diverse group of microorganisms; they may be the cause of some difficulties with PCR. Differential amplification efficiencies of target DNA may be explained by primer degeneracies and poor elongation efficiencies due to the greatly varying G+C content of bacteria (1, 37, 58, 62). To increase the specificity of amplification and decrease the formation of spurious by-products, a "touchdown" PCR can be used (23, 54, 75). In touchdown PCR, the annealing temperature is set 10° C above the final anneal temperature, and is decreased by 1° C every other cycle until the final pre-selected anneal temperature is attained. Additional amplification cycles are then carried out using the final anneal temperature. Because it is possible that touchdown PCR selectively amplifies the most abundant phylotypes, some diversity may be lost in the process of amplification (44). However, the importance of the less dominant microorganisms is debatable, since rare species in a community generally have little effect on the overall flux of energy and matter (62).

A more serious problem associated with the amplification of 16S rDNA is that the ribosomal DNA of some organisms (e.g. *Nanoarchaeota*) is so divergent that PCR with universal primers fails to detect them even from cultured organisms (94). Heteroduplexes, a source of sequence artifact formed when similar strands incorrectly hybridize, are another

problem that can occur in the amplification step. These can be reduced by addition of excess primer (56, 85).

Sequencing of 16S rDNA fragments is commonly followed by a cloning step in order to separate DNA fragments from different microbes. The DNA sequence is then analyzed and compared to known sequences to identify genetically related organisms. Identification at the species level of related microorganisms can be problematic in complex systems because of the similarity of 16S rDNA fragments of some organisms (52, 63).

Although molecular techniques face several obstacles, they remain the best method available for profiling complex communities. Some molecular genetic techniques that have been applied to the study of microbial ecology include terminal restriction-fragment-length polymorphism (22), molecular probes (71), 16S rDNA sequencing libraries (9, 94), random shotgun sequence libraries (44), group-specific fingerprints (87), serial analysis of V1 ribosomal sequence tags (SARST-V1) (61, 100), temperature gradient gel electrophoresis (18, 48, 62), and denaturing gradient gel electrophoresis (56, 63, 75, 99).

### **Denaturing Gradient Gel Electrophoresis (DGGE)**

Combined with PCR amplification of 16S rDNA, DGGE can give a direct display of the predominant organisms in a given sample and is one of the most frequently used methods of molecular community finger-printing (99). Genetic finger-printing techniques provide a pattern of the genetic diversity in a microbial community and are commonly used for the investigation of the temporal and spatial distribution of a bacterial population (57). DGGE has been applied to characterize the microbial communities of several ecosystems including cheese (48, 63, 70), paddy field soil (92), hot springs (28), and medieval wall paintings (33,

75). It has also been used to monitor changes in bacterial populations sampled from different animal digestive systems (52, 72, 78) and to identify fish pathogens (43).

During DGGE, DNA molecules are separated by electrophoresis through a polyacrylamide gel held at 60° C, with a preformed gradient of urea and formamide, two DNA denaturing agents (30). Heating the gel to 60° C has little effect on the polymerization of the gel and has the advantage of causing DNA denaturation at a lower concentration of DNA denaturing agents (30).

DNA molecules denature at different concentrations of denaturants and/or temperatures based on their relative amounts of adenine and thymine. Because there are two hydrogen bonds between adenine and thymine (compared to three between cytosine and guanine), double stranded DNA with a higher A-T percentage requires less heat or denaturant to separate (33). A difference in even one base pair can cause the melting temperature to decrease or increase (28, 29). The basis of DGGE is that the electrophoretic mobility of a partially denatured DNA molecule is practically halted compared to its native helical form. Thus, as the DNA fragment migrates through the gel, its electrophoretic mobility decreases significantly when the critical concentration of DNA denaturants is reached (18, 27, 56). This creates a banding pattern of the various DNA fragments as they reach their melting temperature. Sheffield et al (76) determined that the resolution of the bands could be improved if a 40-base pair G+C-rich sequence, termed the GC-clamp, was attached to the forward primer. Addition of a GC-clamp improves visibility of the bands by preventing the complete disassociation of the two strands (33, 56, 57, 76).

DGGE is a technique that was initially developed as a method for 2D electrophoresis of DNA (31) and was later adjusted to detect single-base mutations in a gel matrix, independent

of size (10, 30, 31). Later, Muyzer and Smalla (57) adopted the method to study the genetic diversity of complex microbial populations. This technique was selected because culture and microscopy techniques cannot identify the majority of naturally occurring microorganisms. Using primers designed to base pair with highly conserved regions of the 16S rDNA of eubacteria, it was eventually determined that the V3 region of 16S rDNA sequence produced the most intense and largest number of bands when run on DGGE (99).

DGGE is capable of detecting microorganisms that make up as little as 1-10% of a population (48, 57, 63). DGGE is considered semiquantitative and the intensity of a band corresponds to the amount of respective template DNA (92). As the intensity varies in a given band position, some conclusions may be made concerning the increase or decrease in population of the respective microorganisms in that position (56, 57, 92). However, due to differential amplification by universal primers and the co-migration of multiple DNA fragments, it should not be assumed that the band intensities from different positions reflect true population abundance when DGGE is used as a community fingerprint (29, 56, 57).

In order to use DGGE to create a DNA fingerprint of a microbial ecosystem, it is essential to verify the purity of a band through sequencing (28). This is important because DNA fragments with varying sequences, but with equal GC-content, have been shown to co-migrate (41, 44, 52, 63, 92). Multiple bands may result from a single organism as well (44, 63, 70). To overcome these issues, cloning is usually combined with DGGE in order to select individual fragments from excised bands for sequencing.



## MATERIALS AND METHODS

### Sampling

Bioreactor samples were collected from the Wade Dairy Farm Reactor, Farr West, Utah. Samples of the influent, effluent, and three vertical levels of the reactor were collected in a large bucket lined with a plastic garbage bag, and approximately forty milliliters were taken from each sample and placed into a separate sterile 50-ml centrifuge tube. Samples were taken on May 17, June 21, and Aug 12, 2005, consecutively. The 50-ml sample tubes were placed in a -20° C freezer for storage.

### DNA Isolation

Genomic DNA was extracted from environmental samples using the PowerSoil™ DNA isolation sample kit (Mo Bio Laboratories, Inc., Solano Beach, CA).

### PCR

The variable V3 region of the 16S rDNA of eubacteria was enzymatically amplified with primers binding to conserved regions of the 16S rDNA genes (56, 75). The nucleotide sequences of the primers were used to amplify the 16S rDNA regions in the various eubacteria species 1, 5'-CGCCCGCCGCGCGCGGGCGGGCGGGGCACGGGGG  
GCCTACGGGAGGCAGCAG-3'; primer 2, 5'-ATTACCG CGGCTGCTGG-3'; Primers 1 and 2 were which correspond to positions 341 to 534 in *E. coli*. Additional primers were designed to amplify the 16S rDNA of archaea (79, 92). Primers 3 and 4 primer 3, 5'-CGCCCGCCGCGCGCGGGCGGGCGGGGCACGGGGGAGGAATTGGCGGG  
GGAGCAC-3'; and primer 4, 5'-GCGGTGTGTGCAAGG AGC-3' amplified the 16S rDNA

region of archaea corresponding to positions 915 to 1386 in *E. coli*. The forward primers 1 and 3 contained a 40-base GC clamp (underlined) to stabilize the melting behavior of the DNA fragments (76). Amplification was performed with a DNA Thermal Cycler (PerkinElmer model 480, Wellesley, MA) in a final volume of 50  $\mu$ l containing: 1X TaqMaster PCR Enhancer (Eppendorf, Westbury, NY), 1X Buffer B (Fisher Scientific, Pittsburgh, PA), 2.5 mM MgCl<sub>2</sub>, 25  $\mu$ M of each dNTP, 2.5 IU Taq DNA polymerase (Fisher), 1  $\mu$ M of the primers, and 1  $\mu$ l cell lysate. The PCR reaction was overlaid with 2 drops of mineral oil (PerkinElmer). In order to increase the specificity of the amplification and decrease the formation of spurious by-products, a "touchdown" PCR was performed (23, 55, 75). Thus, the annealing temperature was initially set to 65° C and decreased by 1° C every other cycle until a touchdown occurs at 55° C, at which temperature 10 additional cycles were carried out. All PCR cycles used a denaturation step of 94° C for 30 seconds, 30 second primer annealing, and elongation at 72° C for 1 min. The PCR reactions began with 94° C for 2 min and ended with an elongation step of 72° C for 7 min, then were chilled to 4° C. All PCR products were analyzed by gel electrophoresis in a 1% (wt/vol) agarose gel and visualized by ethidium bromide staining before DGGE was performed.

## DGGE

Denaturing Gradient Gel Electrophoresis was performed with the CBS Scientific Company DGGE System (Solana Beach, CA). PCR samples were mixed 2 to 1 with 6X Gel-Loading Buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% [wt/vol] sucrose in ddH<sub>2</sub>O), and DGGE was performed on a poly-acrylamide gel (in 1X TAE: 40mM Tris acetate [pH 7.4], 20 mM sodium acetate, 1mM Na<sub>2</sub>-EDTA). Gels were made with 8%

(wt/vol) acrylamide stock solutions (acrylamide-N,N-methylene-bisacrylamide, 27:1[VWR, Bristol, CT]) with denaturing gradients from 25-60% denaturant (100% denaturant corresponds to 7M urea and 40% [vol/vol] formamide deionized). The gels were run at 60° C for 30 min at 150 V, at which time the voltage was increased to 200 V and run for an additional 300 min.

After completion of electrophoresis, the gels were stained by spreading 15 ml of SYBR Gold solution (Molecular Probes Inc., Carlsbad, CA) diluted 1/10,000 over the surface of the gel (14). The gel was incubated in the dark for 10 min, washed repeatedly with ddH<sub>2</sub>O, and carefully removed to be photographed using a UV transilluminator (UVP, Upland, CA). Bands that disappeared or changed in intensity were sampled, as well as many of the more prominent bands on the gel (Fig. 2A and B, and 3). This was done to help identify some of the microorganisms that were present and those that were inhibited. Bands of interest were excised with a clean razor blade and placed in separate microcentrifuge tubes.

### **Cloning and Sequencing of 16S rDNA Fragments**

DNA was eluted from the polyacrylamide gel following the procedure outlined by K. Koo (45) then reamplified by the same procedure described above for touchdown PCR. The newly obtained PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA), cloned into *Escherichia coli* DH5 $\alpha$  using the TOPO TA cloning vector (Invitrogen Corporation, Carlsbad, CA). The vector, which encodes resistance to ampicillin, has a 3'-thymidine residue at the insertion site to facilitate the incorporation of PCR amplicons, which commonly have a single deoxyadenosine added (independent of the template) to the 3' ends of the PCR product (13). The TOPO vectors have topoisomerase I

covalently attached, which increases the efficiency of ligation. The vector was transformed into *E. coli* following the heat-shock procedure outlined by Invitrogen. Five ampicillin-resistant colony forming units were selected from each cloning reaction, were streaked for purity, and were inoculated into Luria-Bertani (LB) broth tubes and grown overnight at 37° C. Plasmids were isolated from tubes showing growth by the alkaline lysis method (40) and the presence of the plasmid was confirmed by electrophoresis in 1% (wt/vol) agarose gels. The forward and reverse strands of each PCR product were sequenced.

Sequences were analyzed using FinchTV (Geospiza, Seattle, WA), GeneWorks (IntelliGenetics, Mt. View, CA), and BLAST (NCBI, Bethesda, MD.), in order to determine the species that is most closely related to that from which the DNA was isolated. The program FinchTV was used to analyze the chromatographs of the sequenced DNA. Next, the sequenced DNA was compared to known segments of 16S rDNA using BLAST in order to find genetically similar microorganisms. GeneWorks was also used to compare the individual strands of sequenced DNA. BLAST (Basic Local Alignment Search Tool), provides a method for rapid searches of the nucleotide database on NCBI. Queries into the database allow for the identification of similar sequences on previously characterized genes.

## RESULTS

### Sample Collection

Samples were collected on three sampling dates (see Table 1). One sample was collected from the influent, bottom reactor port, mid reactor port, top reactor port, and the effluent on each trip.

### Eubacteria Sequences

As shown in Fig. 2A and 2B, eubacteria fingerprints from the various sampling points in the Wade Dairy reactor seem to share the majority of the bands. These microorganisms and their percent identity with the sequenced DNA are listed in Table 2.

Some of the bands selected for cloning and sequence analysis were not present in all of the samples (bands two and three) or because their intensity decreased with passage through the reactor (band six). Band two was identified in a rumen sample, but was not present in any of the samples taken from the anaerobic reactor. The sequences derived from band two revealed microbes closely related to a microorganism from the order Bacteroidales, and *Treponema saccharophilum*, a member of the phylum Spirochaetes that ferments various plant polysaccharides and has been shown to improve overall fiber digestion of some hydrolytic microorganisms (88).

Band three was present in the influent, but quickly disappeared after entering the anaerobic reactor. Sequences obtained from band three revealed it contained fragment derived from at least two species; a close relative of a *Clostridium* sp. and a bacterium that was distantly related to a *Lactobacillus* sp.

TABLE 1. Known parameters of the induced sludge bed reactor on sampling dates (C. S. Hansen, personal communication).

Date	Organic Loading Rate <sup>1</sup>	Dissolved Solids (Influent)	Dissolved Solids (Effluent)	Chemical Oxygen Demand	Consecutive Days Running
17-May-05	140.87 lbs/hr	17.23 g/L	13.51 g/L	NA <sup>2</sup>	5 days
21-Jun-05	113.8 lbs/hr	15.171 g/L	15.058 g/L	NA	18 days
12-Aug-05	88.83 lbs/hr	20.166 g/L	12.297 g/L	6513 mg/L	15 days

<sup>1</sup>Organic loading rate is in terms of volatile solids. <sup>2</sup>NA indicates that information was not available.

Band six was selected for cloning and sequence analysis because it was also present in the influent and, unlike band three, did not completely disappear after entering the reactor. The sequences represented microorganisms that were most closely related to *Lactobacillus*, *Clostridium*, and *Sedimentibacter* species. The DNA fragments from bands three and six were related to the same *Lactobacillus* species, but had a 60 base pair section in the center of the DNA fragment that was divergent from the DNA of *Lactobacillus vitulinus*. When the two fragments were compared, this section was found to be dissimilar, but closely related, indicating that they are likely from different species.

Several of the bands that were excised were selected because of their recurrence among the various samples and their prominence in the gels (intensity). This group includes bands one, four, five, seven, eight nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, and seventeen.

TABLE 2. Species of eubacteria identified in samples collected from an induced blanket reactor.

Band <sup>1</sup>	Closest relative	% Identity	GenBank accession no.
1	<i>Bacteroidetes</i> (phylum)	70	DQ451471
2	<i>Treponema saccharophilum</i>	97	TRPRR16SD
	<i>Bacteroidales</i> (order)	89	AB234433
3	<i>Lactobacillus vitulinus</i>	70	AB210825
	<i>Clostridium</i> sp.	97	DQ232857
4	<i>Cytophagales</i> (family)	91	AY710562
	<i>Clostridium</i> sp.	100	AY685919
5	<i>Bacteroides</i> sp.	93	AY780552
	<i>Prevotella</i> sp.	89	AY331415
6	<i>Lactobacillus vitulinus</i>	70	AB210825
	<i>Clostridium</i> sp.	99	DQ168197
	<i>Sedimentibacter</i> sp.	94	DQ168650
7	<i>Bacteroides</i> sp.	93	AY780552
	<i>Alkalibacterium</i> sp.	94	AY554414
	<i>Lachnobacterium</i> sp.	95	LSP518873
8	<i>Bacteroides</i> sp.	93	AY780552
	<i>Sedimentibacter</i> sp.	93	DQ168650
	<i>Clostridium cellulose</i>	86	CLORG16SH
9	<i>Aequorivita crocea</i>	97	AY027806
	<i>Spirochaeta</i> sp.	100	AF357916
	<i>Desulfuromonas</i> sp.	91	AB189359
	<i>Spirochaeta</i> sp.	95	AJ698092
10	<i>Dehalococcoides</i> sp.	93	DEH431247
11	<i>Bacteroides</i> sp.	94	AY144266
	<i>Flavobacteria</i> sp.	89	DQ168834
12	<i>Tenacibaculum amylolyticus</i>	85	AB032505
	<i>Prevotella</i> sp.	89	AY331415
13	<i>Planctomycetales</i> (order)	93	DQ393189
14	<i>Bacteroides</i> sp.	95	AY780552
15	<i>Actinobacterium</i> (phylum)	77	AY905577
16	<i>Sedimentibacter</i> sp.	98	AY766467
	<i>Spirochaeta</i> sp.	95	AJ698092
	<i>Clostridium</i> sp.	95	AY330127
17	<i>Clostridium</i> sp.	94	AY330127

<sup>1</sup>The numbers of the bands correspond to DNA excised from selected bands on two DGGE gels. Multiple microorganisms listed for a single band indicate the presence of more than one sequence identified.

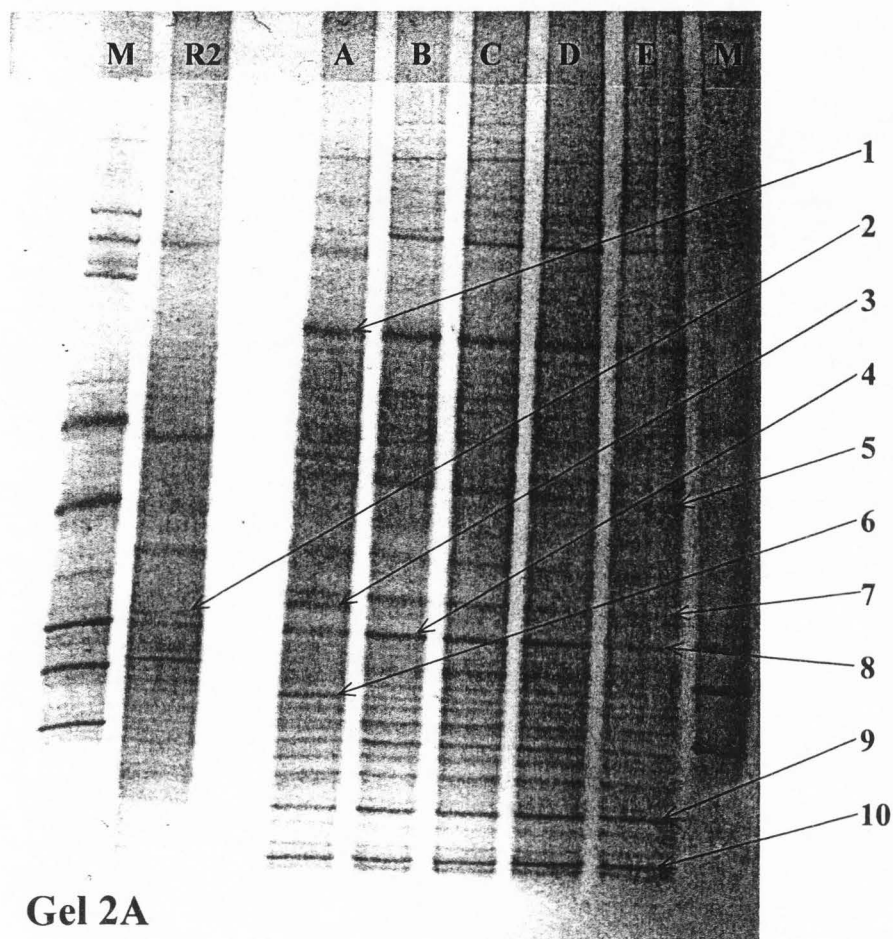
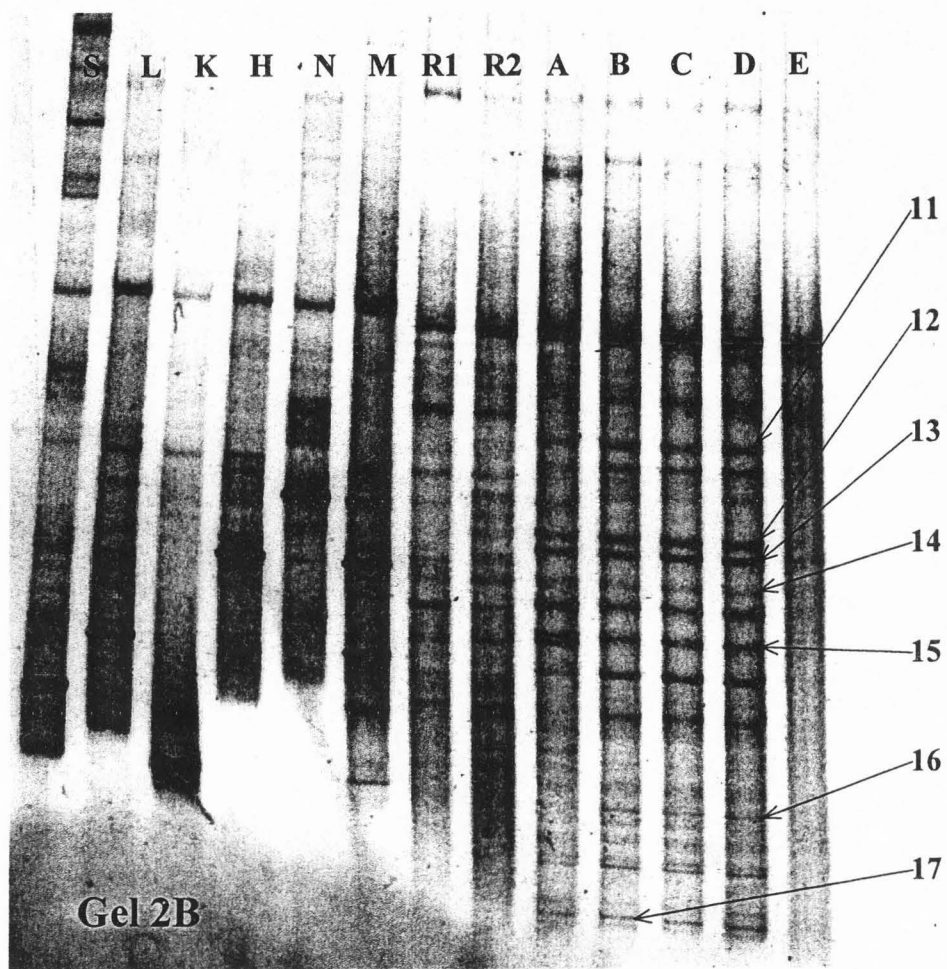


FIG. 2. DGGE analysis of the total bacterial population in samples collected from various ports on the Wade Dairy induced sludge blanket anaerobic reactor (IBR) and from the rumen of fistulated cows. (2A & 2B) Gel images run on different days from identical DNA samples collected from the rumen of two fistulated cows (R1 & R2), and samples collected from the IBR. Samples consist of the influent of the IBR (A), lowest testing port on the reactor (B), testing port near the middle (15 feet high) (C), Highest testing port (D), effluent of the reactor (E). In order to create a marker for the DGGE gels, DNA was extracted from pure cultures of *Escherichia coli* DH5 $\alpha$  (S), *Lactococcus lactis* sp. *lactis* SL156 (L), *Lactobacillus casei* 334 (K), *Lactobacillus helveticus* CNRZ32 (H), and *Listeria monocytogenes* N1-227 (N). The DNA from the various bacteria was combined in equal proportions to make a marker (M).





Based on intensity, populations that generated bands one, four, eight, eleven, and sixteen appear to increase in the reactor. Band one contained DNA of a bacterium that was related to microorganisms in the phylum Bacteroidetes. The DNA from the two microorganisms isolated in band four were closely associated with a species of *Clostridia* and a member of the family Cytophagales. Although band eight migrated to the same position as band four, the sequences derived from clones resulted in three different species of bacteria, *Bacteroides*, *Sedimentibacter*, and *Clostridium*. Therefore, the presence of even more microorganisms may be identified by increasing the amount of sequences from bands at this position in the gel (37).

Band eleven revealed the presence of organisms closely related to *Bacteroides* and *Flavobacterium*, while band sixteen bore sequences resembling species of *Sedimentibacter*, *Spirochaeta*, and *Clostridium*. The organisms that were most closely associated from the four bands with increasing intensity (one, four, eight, eleven, and sixteen) included various species of anaerobes and facultative anaerobes (see Table 2). The genera of *Bacteroides*, *Clostridium*, and *Sedimentibacter* were well represented. This was expected given the temperature and anaerobic environment of the reactor.

Three of the excised bands (seven, nine, and ten) had the same intensity in each of the levels sampled. Band seven yielded sequences that were closely related to bacteria in the genera *Bacteroides*, *Alkalibacterium*, and *Lachnobacterium*. Sequences obtained from band nine revealed two distantly related species of *Spirochaeta*, as well as organisms closely related to *Aequorivita crocea* and *Desulfuromonas* sp. Band ten contained a DNA fragment derived from a bacterium related to a *Dehalococcoides* species.

Bands five, twelve, thirteen, and fifteen were selected for analysis due to their prominence on the gel. Bands five and thirteen migrated to the same position on the gels run on separate days (Fig. 2A and 2B) and revealed close relatives of species of *Bacteroides* and *Prevotella*, and a microorganism from the order Planctomycetales, respectively. The DNA sequenced from these bands was similar to sequences obtained from *Tenacibaculum amylolyticus* and a *Prevotella* species from band twelve, and another related to the phylum Actinobacterium from band fifteen. Bands fourteen and seventeen were less conspicuous and yielded products derived from microbes related to species of *Bacteroides* and *Clostridium*, respectively.

TABLE 3. Species of archaea identified in samples collected from an induced blanket reactor.

Band <sup>1</sup>	Closest relative	% Identity	GenBank accession no.
1	<i>Methanosarcina barkeri</i>	97	CP000099
	<i>Methanospirillum hungatei</i>	98	CP000254
	<i>Methanosarcina mazei</i>	94	AB065295
2	<i>Methanosarcina barkeri</i>	96	CP000099
3	<i>Methanoculleus palmaeoli</i>	99	NPY16382
	<i>Methanosarcina barkeri</i>	96	CP000099
4	<i>Methanosarcina barkeri</i>	96	CP000099
5	<i>Methanosarcina barkeri</i>	96	CP000099
6	<i>Methanobrevibacter</i> sp.	100	AY351473
7	<i>Methanobrevibacter</i> sp.	98	AY351473

<sup>1</sup>The numbers of the bands refer to those in Fig. 3. Multiple microorganisms listed for a single band indicate the presence of more than one sequence identified.

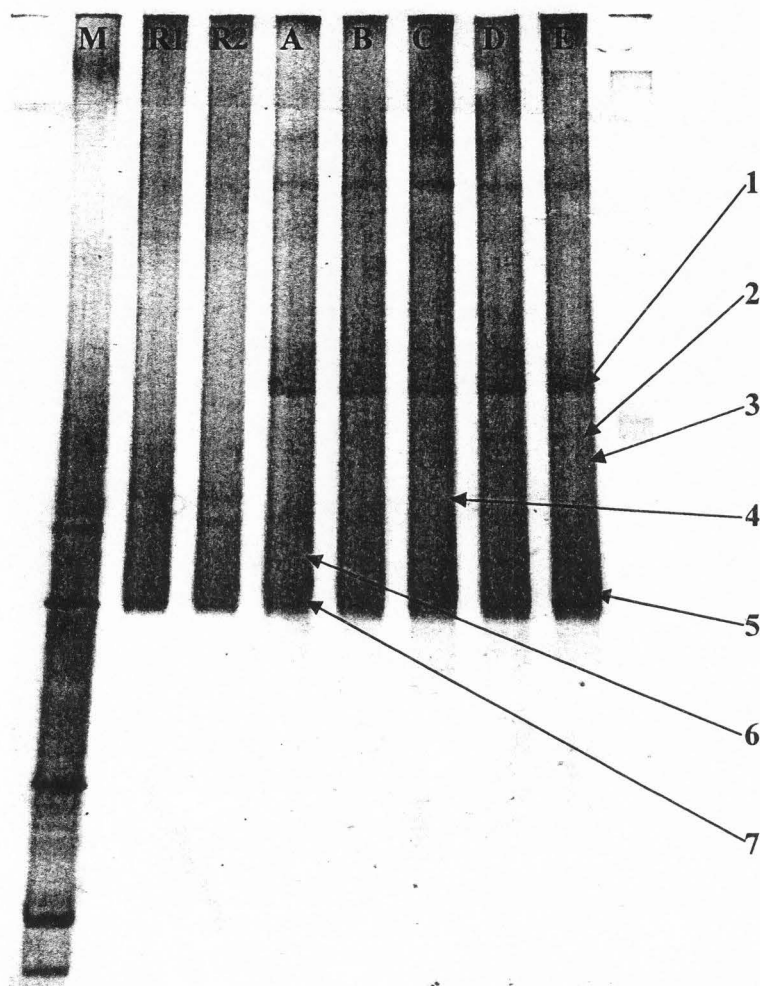


FIG. 3. DGGE analysis of the total archaeal population in samples collected from various ports on the Wade Dairy induced sludge blanket anaerobic reactor (IBR) and from the rumen of fistulated cows. Gel images from samples collected from the rumen of different fistulated cows after DNA extraction (R1 & R2), and from the influent of the IBR (A), lowest testing port on the reactor (B), testing port near the middle (15 feet high) (C), Highest testing port (D), effluent of the reactor (E). DNA from various bacteria was combined in equal proportions to make a marker (M)

### Archaea Sequences

As expected, DGGE with archaeal primers bands that contained 16S rDNA fragments from various species of methanogens (see Fig. 3). The species of archaeal and their percent identity with the sequenced DNA are listed in Table 3.

Band one was one of the more prominent bands and it contained DNA derived from archaea related to different species of methanogens, including *Methanosarcina barkeri*, *Methanospirillum hungatei*, and *Methanosarcina mazei*. *Methanosarcina barkeri* DNA was also recovered from DNA isolated from bands two, three, four, and five. Another sequence acquired from band three was similar to the 16S rDNA of *Methanoculleus palmaeoli*. Bands six and seven contained homologous 16S rDNA from a species of *Methanobrevibacter*. *Methanobrevibacter* is solely a hydrotropic methanogen, consumes hydrogen and carbon dioxide to synthesize methane. *Methanosarcina* is hydrotropic as well as acetotrophic, converts acetate to methane (5). *Methanoculleus* and *Methanospirillum* species are closely related to *Methanosarcina* species, and share similar metabolic abilities.

### Comparison of Eubacteria Fingerprints

Molecular fingerprint data for a bacterial community is often a useful way to quickly determine changes that occur in a population over relatively short periods of time or to identify microorganisms in the population. Figures 4A and 4B show molecular fingerprints from PCR-DGGE analysis of samples taken at different days in the start-up process (see Table 1). The first samples were taken 5 days after the IBR started running and band one is only present in these samples. The converse is true with band twelve, which is only present in samples taken on the second and third dates, which were taken after the reactor had been

running for 18 and 15 days, respectively. Band eleven which is not present in the influent of any of the samples; is derived of DNA from two anaerobes that are able to grow in the reactor, but, evidently, not out of it. Bands six and three revealed two species related to *Lactobacillus*, two species of *Clostridium*, and one of *Sedimentibacter*. These organisms are present in the holding pools, but the band density appears to decrease in intensity with passage through the reactor.

Many of the bands are visible in each sampling day. Some of the organisms that appear to be present in each sample, yielded bands four, eight, ten, sixteen, and seventeen. These bands represent various species of anaerobes, including species of *Clostridium*, *Bacteroides*, *Sedimentibacter*, *Spirochaeta*, *Dehalococcoides*, and a member of the family *Cytophagales*. Bands five, seven, thirteen, and fifteen are also visible in every sample, although they appear to be less concentrated in the first sampling period. These bands were derived of DNA from a member of the order *Planctomycetales*, another from the phylum *Actinobacterium*, and species of *Bacteroides*, *Prevotella*, *Alkalibacterium*, *Lachnobacterium*. Species of *Aequorivita*, *Spirocheata*, *Desulfuromonas*, and *Bacteroides* were recovered from bands nine and fourteen. These two bands appear faintly in all of the samples.

### **Comparison of Archaeal Fingerprints**

The influent from samples one and two (see Table 1) have two bands that match up to the DNA from band six which are derived from a species of *Methanobrevibacter* (see Fig. 5). These bands decrease in intensity as the samples move up the reactor. This may indicate that *Methanobrevibacter* species are out-competed or are much more concentrated in the rumen. The dense band which seems to compromise bands one and three is more concentrated in the

effluent and middle and highest ports. This is consistent with the designed function of stratified layers in the reactor. It appears that the diversity of the methanogens decreases as the waste moves through the IBR, indicated by the decrease in bands. On the other hand, the increase in density and intensity of the main (dense) band may describe an increase in the concentration of methanogens with their passage through the reactor.



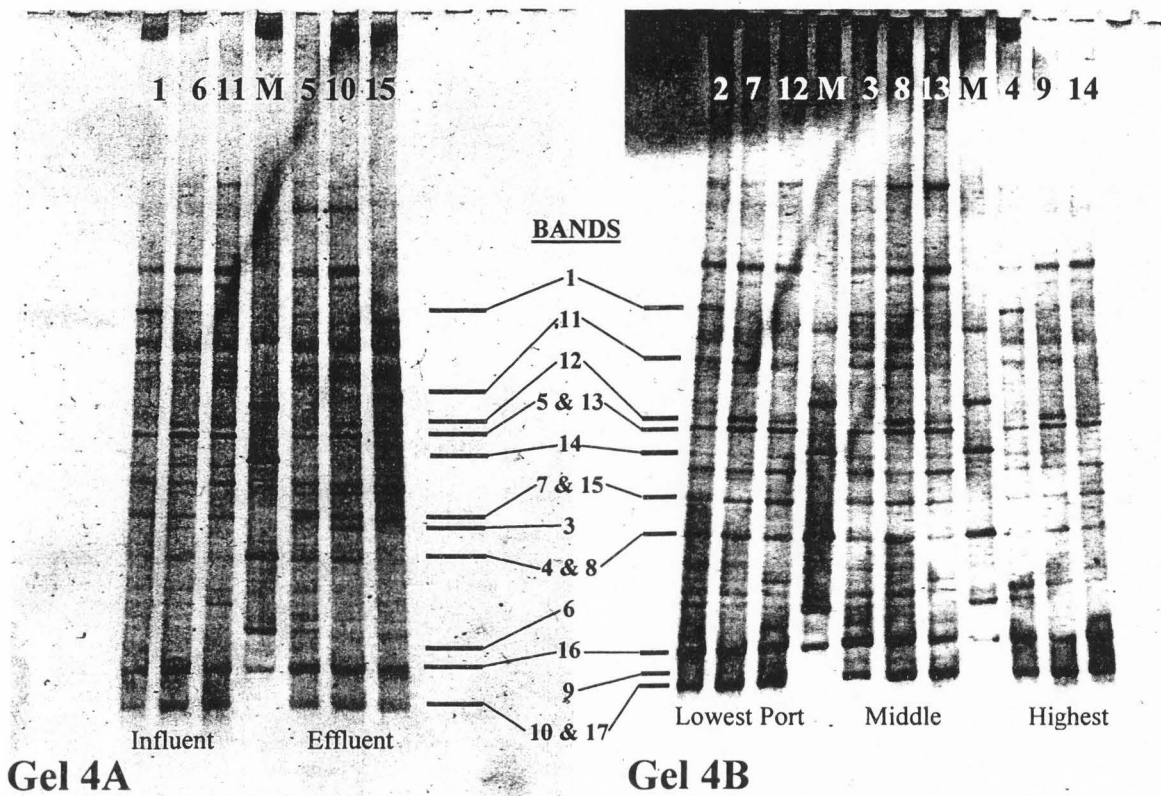


FIG. 4. DGGE comparison of 16S rDNA eubacteria fingerprints from different sampling dates. The samples are set-up in such a way as to allow <sup>a</sup>side-by-side comparison of the various points in the Wade Dairy induced sludge blanket anaerobic reactor (IBR).

<sup>a</sup>The May 17<sup>th</sup> samples are listed as 1-5, June 21<sup>st</sup> as 6-10, and August 12<sup>th</sup> as 11-15. (A) Gel images comparing the influent (1, 6, 11) and effluent of the IBR (5, 10, 15). (B) Gel images comparing the lowest testing port on the reactor (2, 7, 12), testing port near the middle (15 feet high) (3, 8, 13), Highest testing port (4, 9, 14). DNA from various bacteria was combined in equal proportions to make a marker (M).

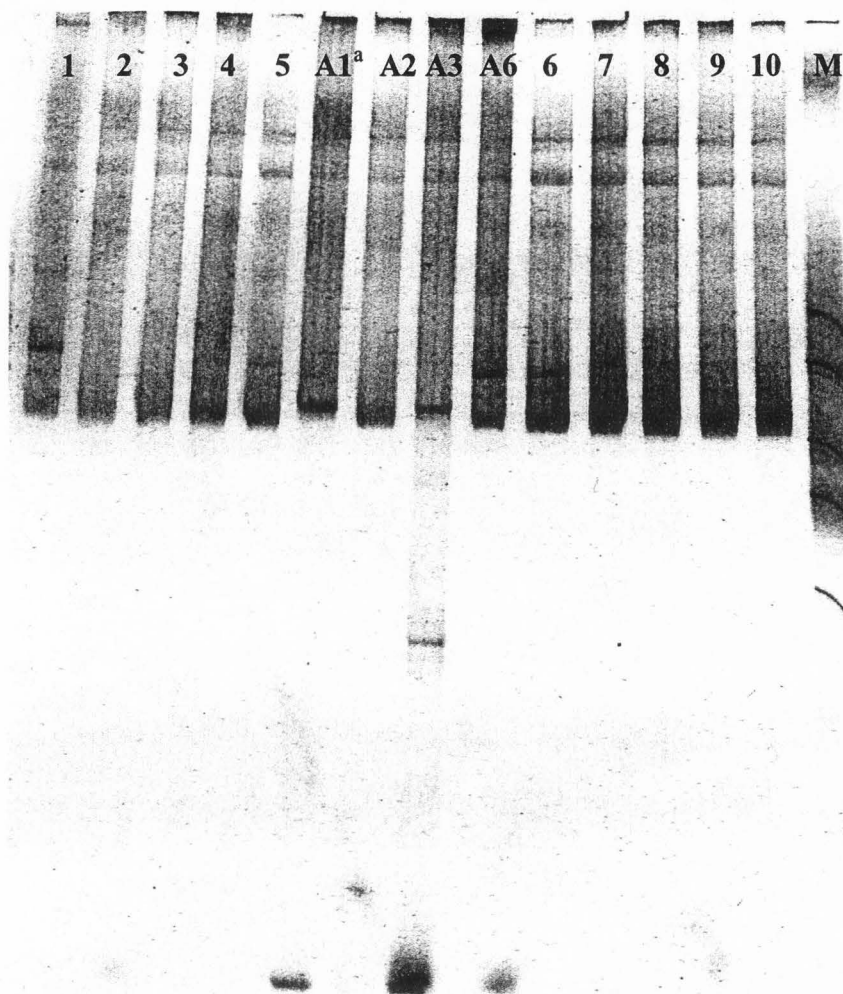


FIG. 5. DGGE comparison of 16S rDNA archaea fingerprints from different dates. May 17th samples from the IBR are listed as 1-5, and June 21st as 6-10. Gel images comparing the influent (1, 6), lowest testing port on the reactor (2, 7), testing port near the middle (15 feet high) (3, 8), Highest testing port (4, 9), and effluent of the IBR (5, 10). DNA from various bacteria was combined in equal proportions to make a marker (M).

<sup>a</sup>The numbers preceded by the letter A, indicate DNA taken from excised bands.

## DISCUSSION

In this study, bacteria and archaea in an induced blanket reactor were identified by a non-culture technique, DGGE. DGGE is an effective tool because it is a reliable, reproducible, rapid, and inexpensive method for the analysis of a variety of ecosystems (33, 44, 55, 57).

Several different species of microorganisms associated with anaerobic environments and waste degradation were identified in the IBR community, including *Dehalococcoides*, *Clostridia*, *Desulfuromonas*, and *Bacteroides* species. One of the goals of this research has been to describe the microorganisms present in the IBR, so that the niche of each may be identified. Hydrolytic, fermentative, and methanogenic microorganisms are the three basic groups necessary for complete anaerobic degradation of plant material and the production of methane in the IBR.

Hydrolytic species are responsible for the breakdown of the complex plant polysaccharides that remain after passage through the bovine gastrointestinal tract. The hydrolytic organisms fall into two categories: primary fermenters such as *Fibrobacter* (formerly *Bacteroides*) *succinogens*, *Ruminococcus albus* and *flavefaciens*, whose only source of energy involves the break down of cellulose, and secondary fermenters (4, 80, 88, 93). Secondary fermenters include species such as *Butyrivibrio fibrisolvens* and various species of *Clostridium*, which are able to derive energy from more than just cellulose (77, 93, 102). Some of these organisms can also break down protein, especially species of *Clostridium* (36). *Sedimentibacter* is closely related to *Clostridium* but is unable to ferment

carbohydrates. Instead growth is supported by the fermentation of pyruvate and amino acids (13).

*Clostridium* sequences found in bands six, sixteen, and seventeen closely resemble the 16S rDNA of *Clostridium longisporum*, a known cellulolytic organism (93). Bands sixteen and seventeen are consistent in all of the samples and may indicate that cellulolytic species of *Clostridium* are present throughout the process.

Many of the organisms that are associated with the fermentation of available carbohydrates are also effective at complex carbohydrate degradation. Species identified in the IBR that fit in this classification include *Tenacibaculum amylolyticum* and the closely related organism from the *Cytophagales* family, as well as species of *Prevotella*, and *Flavobacterium* (83, 90, 98). *Tenacibaculum amylolyticum*, *Aequorivita crocea*, and some species of *Flavobacterium*, *Planctomyces*, and *Spirochaeta* are capable of hydrolyzing proteins and some polysaccharides, such as starch, and/or gelatin (11, 24, 32, 83, 90, 98). *Prevotella* species have the ability to degrade several plant polysaccharides, starch and hemicellulose for example, and may have synergistic interactions with cellulolytic bacteria in hemicellulose and pectin degradation (44, 46).

Some of these organisms may require more time to become established in the reactor. Close relatives of *Prevotella*, *Planctomyces*, and *Tenacibaculum* species, for example, (recovered as bands five and twelve) appear to increase in concentration in the reactors, especially in samples taken from longer running times. *Planctomyces* have been identified in various environments by their ability to grow on N-acetylglucosamine, whether or not this is part of the role played by this organism in the IBR is unknown (32). The bands seem to indicate that species of *Clostridium*, *Flavobacterium*, *Aequorivita*, and *Spirorochaeta* appear

to be consistent throughout the process. The relative faintness of the bands for species of *Aequorivita* and *Spirochaeta* may suggest that these organisms do not make up a significant portion of the reactor microbiota.

Many of the organisms identified in the IBR are fermenters of mono- and disaccharides and are largely responsible for the production of the volatile fatty acids (VFA) that fuel methanogenesis. These organisms include species of *Alkalibacterium*, *Spirochaeta*, *Lachnobacterium*, *Clostridium*, *Flavobacterium*, and *Bacteroides* (6, 24, 59, 73, 96, 101, 103). While acetate is the most predominant fermentation product, species of *Alkalibacterium* and *Lachnobacterium* also produce lactate as a major end product (59, 96, 101).

Due to the reduction of band intensity in the effluent and influent it appears that some species of *Prevotella*, *Alkalibacterium*, *Lachnobacterium*, and *Bacteroides* are unable to survive in significant numbers outside of the reactor. This is likely the result of contact with oxygen.

Methanogens make up the last step of the process and are involved in the conversion of hydrogen, carbon dioxide, and acetate to methane. The role of methanogens in this process is vital to the maintenance of the anaerobic process, as they are responsible for the removal of hydrogen and acetate from the reactor, thus maintaining the pH of the environment (4, 73). Methanogens also form syntrophic relationships with various fermenters by supplying these organisms with essential amino acids in exchange for the supply of the necessary substrate (4). All of the methanogens identified in this are able to convert hydrogen and carbon dioxide to methane (see Table 2), but species of *Methanosarcina* are out-competed for hydrogen by most methanogens (4, 5). *Methanosarcina* has a relatively low specific growth

rate on hydrogen and must therefore derive much of its energy from an additional source (4, 5).

*Methanosarcina mazei* and *barkeri* are the only methanogens detected in the reactor that are able to grow on acetate (4, 5, 81). While *Methanosarcina* is unable to compete with other methanogens for hydrogen; it does not appear to decrease in concentration (4).

*Methanobrevibacter* have previously been shown to establish early and may not be able to compete with *Methanospirillum* and *Methanoculleus* species that perform the same function (21, 79).

Two microorganisms identified in the reactor are likely to compete with methanogens for hydrogen and acetate. Species of *Desulfuromonas* oxidize acetate, lactate, succinate, and other VFA with the reduction of elemental sulfur or ferric oxide (17). *Dehalococcoides* species require hydrogen to reduce chemoorganic compounds (e.g. tetrachlorethene, trichloroethene) for their metabolism (82). The presence of both of these organisms is constant throughout the process, but band nine containing *Desulfuromonas* DNA does not appear to be very intense.

Anaerobic digestion is a very resilient process, but its successful application for industrial use may be contingent on reducing interference from outside influence. Animals receiving antibiotics should be separated from the others, in order to insure that the antibiotic containing feces are not pumped into the anaerobic reactor. When antibiotics are present in the influent of anaerobic digestion the production of VFA and methane are decreased (25).

Antibiotics are not the only substance that can affect methane production in the influent. Sulfate-reducing bacteria can effectively compete with methanogens for hydrogen and acetate when sulfur levels are higher (49, 65, 74). In fact, methane-oxidizing archaea

(*Methanosarcinales*) appear to partner with a hydrogen-scavenging, sulfate-reducing partner in the presence of sulfur (64, 67). Molybdate, a micronutrient used for nitrogen fixation, can overwhelm the microbial uptake system when present in high concentrations (71). Addition of molybdate was shown to reduce the amount of sulfate reduced and increase the rate of methane production (49).

Volatile fatty acids are important to the maintenance and establishment of methanogens in an anaerobic system. Addition of easily degradable matter in the form of VFA improves the general behavior of the process due to an increased amount and activity of VFA degraders (68). Also, addition of acetate to an anaerobic digester resulted in significantly better phosphate removal from animal waste (9). Addition of volatile fatty acids may therefore be one solution to decreasing the amount of start-up time for a reactor or may help to improve the removal of pollutants from a reactor.

Additional research into the microbial ecology of an anaerobic system such as an induced blanket reactor may help to identify other areas of improvement for the system. In any community, the number of species of organisms identified increases with sampling effort until all types are observed (37). While DGGE is an effective tool for rapid identification of the major organisms in any environment, it is not useful for determining the quantity of each organism present and is limited to more abundant organisms. New techniques such as serial analysis of ribosomal sequence tags (SARST) appear to have more potential for quantitative analysis of the population (61, 100).

This technique (SARST) uses universal primers with short sequences with recognition sites for restriction enzymes attached to the 5' end. Restriction enzymes are bacterial DNases that cleave DNA at a recognized position. Many of these restriction enzymes leave a short

segment of single stranded DNA (sticky ends) on the end that will readily hybridize with a similar strand. After PCR amplification of the short segment of 16S rDNA, restriction enzymes are added to the DNA fragments. The restriction enzymes cleave the short sequences from the DNA fragment leaving a sticky end. These sticky ends on the amplified fragments allow for the hybridization of the community 16S rDNA segments. This new sequence of DNA may contain DNA fragments from several species. These fragments are then loaded into a cloning vector, often with several fragments combined in one plasmid. This decreases the number of clones that are necessary for large-scale identification of the microbiota present in an environment. With an average of six fragments hybridized together on a single plasmid; sequencing of plasmids from one hundred clones could provide a very detailed picture of the individual organisms present and the percentage of the population that they make up (61, 100). While SARST would still be limited by PCR bias, it would be an excellent method for determining the relative abundance of the key players in the population, as well as identifying all of the lesser organisms.

Another molecular technique that has a tremendous amount of potential for revealing the microbial ecology of a given population is the use of functional genes as molecular markers to perceive metabolic activity (42, 56). Primers may be designed that will amplify genes relating to an important function, cellulase genes for example. These primers could be applied to conserved regions of DNA and applied to a DGGE system in order to separate the amplified DNA fragments. Because activity in the reactor is only inferred; functional genes would provide validation that certain organisms were present to perform a given function in an ecosystem (95).



The sequencing of microorganisms from environmental samples consistently produces of the emergence of these new microorganisms, culture-based techniques will continue to be an important means of assigning metabolic function to unknown species (12, 55, 57, 58, 74, 94). DGGE may be useful for the isolation of some microorganisms. DGGE can be used to identify unknown organisms in a given environment. Once these organisms are identified, an appropriate media can be used to select and enrich for this species. Teske et al. (84) were able to use DGGE to identify two symbiotic organisms in culture; this helped to determine the choice of selective culture conditions to separate both target organisms.

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