Genomic and Proteomic Analysis of Microbial Function in the Gastrointestinal Tract of Ruminants*
- Review -

Bryan A. White[1] and Mark Morrison[2]
Departments of Animal Sciences and Veterinary Pathobiology, and the Division of Nutritional Sciences
University of Illinois at Urbana-Champaign 1207 West Gregory Drive, Urbana, IL 61801, USA

ABSTRACT : Rumen microbiology research has undergone several evolutionary steps: the isolation and nutritional characterization of readily cultivated microbes; followed by the cloning and sequence analysis of individual genes relevant to key digestive processes; through to the use of small subunit ribosomal RNA (SSU rRNA) sequences for a cultivation-independent examination of microbial diversity. Our knowledge of rumen microbiology has expanded as a result, but the translation of this information into productive alterations of ruminal function has been rather limited. For instance, the cloning and characterization of cellulase genes in *Escherichia coli* has yielded some valuable information about this complex enzyme system in ruminal bacteria. SSU rRNA analyses have also confirmed that a considerable amount of the microbial diversity in the rumen is not represented in existing culture collections. However, we still have little idea of whether the key, and potentially rate-limiting, gene products and (or) microbial interactions have been identified. Technologies allowing high throughput nucleotide and protein sequence analysis have led to the emergence of two new fields of investigation, genomics and proteomics. Both disciplines can be further subdivided into functional and comparative lines of investigation. The massive accumulation of microbial DNA and protein sequence data, including complete genome sequences, is revolutionizing the way we examine microbial physiology and diversity. We describe here some examples of our use of genomics- and proteomics-based methods, to analyze the cellulase system of *Ruminococcus flavefaciens* FD-1 and explore the genome of *Ruminococcus albus* 8. At Illinois, we are using bacterial artificial chromosome (BAC) vectors to create libraries containing large (>75 kbases), contiguous segments of DNA from *R. flavefaciens* FD-1. Considering that every bacterium is not a candidate for whole genome sequencing, BAC libraries offer an attractive, alternative method to perform physical and functional analyses of a bacterium's genome. Our first plan is to use these BAC clones to determine whether or not cellulases and accessory genes in *R. flavefaciens* exist in clusters of orthologous genes (COGs). Proteomics is also being used to complement the BAC library/DNA sequencing approach. Proteins differentially expressed in response to carbon source are being identified by 2-D SDS-PAGE, followed by in-gel-digests and peptide mass mapping by MALDI-TOF Mass Spectrometry, as well as peptide sequencing by Edman degradation. At Ohio State, we have used a combination of functional proteomics, mutational analysis and differential display RT-PCR to obtain evidence suggesting that in addition to a cellulosome-like mechanism, *R. albus* 8 possesses other mechanisms for adhesion to plant surfaces. Genome walking on either side of these differentially expressed transcripts has also resulted in two interesting observations: i) a relatively large number of genes with no matches in the current databases and; ii) the identification of genes with a high level of sequence identity to those identified, until now, in the archaebacteria. Genomics and proteomics will also accelerate our understanding of microbial interactions, and allow a greater degree of *in situ* analyses in the future. The challenge is to utilize genomics and proteomics to improve our fundamental understanding of microbial physiology, diversity and ecology, and overcome constraints to ruminal function. (*Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 6 : 880-884*)

Key Words : *Ruminococcus*, Cellulase, Genomics, Proteomics

INTRODUCTION

The rumen, the most extensively studied gut community, contains large numbers of bacteria (up to 10^{11} viable cells per ml comprising 200 species), ciliate protozoa (10^4-10^6 per ml spread over 25 genera), anaerobic rumen fungi (zoospore population densities of 10^3-10^5 per ml divided into 4 genera) and bacteriophage (10^7-10^9 particles per ml) (Orpin and Joblin, 1988; Stewart and Bryant, 1988; Williams and Coleman, 1988; Klieve and Swain, 1993; Hespell et al., 1997). The utilization of plant materials by ruminants is dependent on the microbial digestion of plant structural polysaccharides (cellulose and non-cellulosic structural polysaccharides) that account for over 50% of the weight of plant tissue. Thus the rumen environment has selected for and accelerated the evolution of microorganisms which can use cellulose as a source of carbon and energy. The animal is also dependent on its microbiota to convert and degrade these complex polysaccharides into simple products that can be utilized for energy and growth.

* This paper was presented at the 3rd Joint Symposium of Japan and Korea on Rumen Metalbolism and Physiology which was held on October 1~3, 2000 in Miyagaki, Japan.
[1] Address reprint request to Bryan A. White. Tel: +217-333-2091, FAX: +217-333-8804, E-mail: b-white2@uiuc.edu.
[2] Departments of Animal Sciences and Microbiology, The Ohio State University, 230 Plumb Hall, 2027 Coffey Road, Columbus, OH 43210-1094, USA. Tel: +614-688-5399, FAX: +614-292-7116, E-mail: morrison.234@osu.edu.

Within the rumen, the microbial digestion of plant polysaccharides yields mono- and di-saccharides that are further fermented principally to acetate, propionate, and butyrate, as with relatively small amounts of carbon sequestered as microbial cells. However, the degradation of plant cell wall material to products that can be utilized by the animal is slow and incomplete. Therefore, if one understands the complete degradative machinery and regulatory mechanisms involved at the microbial level then it would be possible to increase plant cell wall degradation in order to increase overall feedstuff utilization by the host animal.

It is widely accepted that the cellulases of ruminal bacteria remain cell associated, probably in complexes analogous to the Clostridial cellulosomes. The recalcitrance of rumen cellulolytic bacteria such as *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* to genetic manipulation has made their study difficult. Classical genetic approaches such as rDNA technology and molecular biology have been applied to the analysis of cellulolytic enzymes from ruminal bacteria (Forsberg et al., 1997; Vercoe and White, 1997). This has resulted in the isolation and characterization of individual cellulase genes. There is no doubt that the cloning of ß-glucanase genes in *Escherichia coli* has yielded some valuable information about this complex enzyme system, and has provided some tools that may ultimately lead to a better understanding of the regulation of the genes and gene products responsible for the efficient utilization of complex carbohydrates. However, the use of these classical genetic tools has had its limitations (Vercoe and White, 1997). The current sequence database is limited to information related only to structural genes, and little is known about the regulation of gene expression, as well as the synthesis and assembly of these complex enzyme systems (Forsberg et al., 1997; Vercoe and White, 1997). Furthermore, the complete cellulase complement of enzymes required for complete plant cell wall hydrolysis has not been elucidated for any ruminal microorganism. Finally, the development of host-vector systems for cellulolytic ruminal bacteria has been largely unsuccessful (Vercoe and White, 1997; White, 1998). Thus, classical mutational studies and the generation of hyper-cellulolytic strains of ruminal bacteria has not been possible. Indeed, even with extensive attempts, developments in the area of ruminal bacterial enzymology and genetics have reached a plateau. Two questions that require resolution are : 1.) are ruminal cellulases organized into a higher order structure and if so, how; and 2.) what are the regulatory systems that are responsible for the differential expression of genes in response to cellulose? The lack of an extensive database of cellulase gene sequences, and the lack of a viable genetic system for these organisms has severely limited progress in this area.

The massive accumulation of microbial DNA sequences, including complete genome sequences, is revolutionizing the genetic analysis of microbes. Classical genetic techniques based on model organisms such as *Escherichia coli* and *Bacillus subtilis* and the use of host vector systems are no longer the only tools that can be used to investigate gene structure, expression and function of diverse bacteria. Indeed, promising technologies, based on functional genomics and proteomics, are emerging which allow one to rapidly investigate the biological features of any organism, without prior development of a specific genetic system for that organism. This paper will review our current genomic- and proteomic-based research approach towards ruminal microorganisms. Results from this approach will accelerate our understanding of the physiology, genetics and regulatory mechanisms of these important ruminal microbes.

PROTEOMIC-BASED RESEARCH

Proteomics-based research centers on the proteome, the protein complement of the genome. Relative to the fixed information base of the genome, the proteome is a dynamic feature subject to changing environmental cues and contexts (Cordwell, et al., 1999). In essence, proteomics has been practiced for many years, since the development of two-dimensional protein gel electrophoresis (2D-PAGE) techniques pioneered by OFarrell (1975). However, the development and implementation of mass spectrometric techniques to protein analysis, as well as the availability of a functional nucleotide sequence (e.g. ESTs; expressed sequence tags) are major technological advances which have increased the recovery of information. By traditional methods, the amount of resolvable protein on a gel has been at least one order of magnitude below the amount needed for Edman analysis (Williams, 1999). Introduction of mass spectrometry and EST data now allow presumptive identification of a protein with less material and less amino acid sequence data.

The proteomic approach can be divided into four general stages: sample preparation to gel imaging, image to spot digestion, digest to database searching, and search results to data analysis and archiving (Quadroni and James, 1999). The central feature maintained from previous protein analysis approaches is two-dimensional gel electrophoresis (2D-PAGE; Hatzimanikatis et al., 1999). Large-format gels are highly reproducible and staining by Coomassie-blue or silver-staining allow quantification over a wide range. Until recently the major impediment to identification were low-throughput techniques, relying either on the aforementioned Edman sequencing or Western blotting. Inroads in robotic automation of the process would

greatly enhance the informational turnaround in proteomic efforts and engender informational maps akin to genome sequence archives. These proteomic databases would be in the form of quantitative maps of protein expression and "physical maps" of protein localization within a biological system. An emphasis on the message portion of biological entities (mRNA or cDNA) has been due to powerful molecular biology techniques that have no complement for the functional portion (protein). Automation is now balancing this inequity.

Proteomics applications with *Ruminococcus flavefaciens* FD-1

We have prepared protein preparations (intracellular and extracellular proteins from cellobiose or cellulose grown cells) which were then sent to the Protein Services Laboratory in the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. There, the protein preparations were subjected to 2D-PAGE. These analyses indicated the appearance of at least 5 new polypeptides for *R. flavefaciens* FD-1 grown using media containing either cellulose as the carbon source. The differentially expressed proteins have been subjected to in-gel-digests and peptide mass mapping/ data base by MALDI-TOF Mass Spectroscopy. We are now comparing the protein sequences of these differentially expressed protein identified to our current cellulase clones (*celA*, *celB*, *celD* and *celE*; Vercoe et al., 1995b; Vercoe et al., 1995a; Wang and Thomson, 1990; Wang and Thomson, 1992; Wang et al., 1993) as well as with other appropriate databases. Newly identified or unknown proteins will be further analyzed by generating oligonucleotide probes for use in probing our clone libraries.

Proteomics applications with *Prevotella bryantii*

This bacterium is best known for its abilities to degrade xylans and proteins. Recent success has been obtained with the use of chemical mutagenesis strategies that have created mutant strains of *P. bryantii* that are defective in either gingipain-like peptidase activity (Madeira et al., 1998), peptide utilization (Peng and Morrison, 1997) or glutamate dehydrogenase activity (Wen and Morrison, unpublished data). In the former two instances, a proteomics-based approach has been a valuable comparative tool, to identify protein(s) lost or modified in the mutant strains. Using these procedures, we have been able to identify candidate proteins possessing gingipain-like enzyme activity (Madeira and Morrison, unpublished data) and amino-terminal sequence data has been obtained for these proteins by Edman degradation procedures. Such information will be used to develop degenerate oligonucleotide primers to support a reverse genetics scheme to clone and isolate the gene(s) encoding these interesting groups of rumen bacterial peptidases.

In conclusion, the enhanced sensitivity of protein stains, improved resources for 2D-PAGE, and the integration of mass-spectrometric procedures, have elevated the ability of researchers to proceed with a proteomics-based approaches. Coupled with the extensive genetic databases and computational software for analyses, it is now possible to take a more global approach to examining rumen bacterial physiology, including the creation and analysis of mutant ruminal bacterial. The identification of gene products coordinating key metabolic or regulatory functions are enhanced, by the technological advances that have created the field of proteomics.

GENOMIC-BASED RESEARCH

Genomics refers to a suite of functional and comparative methods that capitalize on having the entire genome of a particular organism sequenced, either completely with no gaps, or to a high level of coverage that reduces gap size to a minimal amount. Genomics offers the researcher the ability to rapidly investigate all of the biological features of any organism of choice, without prior development of a specific genetic system for that organism. High throughput genome sequencing offers the potential to obtain a complete blueprint for the lifestyle of a specific microbe, and to assess its genetic potential in a comparative fashion. Scientists are already using comparative and functional genomics to answer difficult but fundamental questions in microbiology (e. g. Koonin and Galperin, 1997; Bork et al., 1998; de Saizieu et al., 1998; Paulsen et al., 1998; Kim et al., 1999). Clearly, not every microbial species is a candidate for whole genome sequencing, nor is it affordable, or possible, to develop genetic tools for each species of interest. However, there are other approaches to functional and comparative genomics that do not require complete genome sequence information. One approach to functional genomics relies on the utility of bacterial artificial chromosome (BAC) libraries for obtaining large, contiguous segments of DNA within the genome. Another approach uses a combination of functional proteomics, mutational analysis and differential display RT-PCR to obtain genomic information. In addition, genome walking on either side of these differentially expressed transcripts can also be used to further investigate genome sequence organization and content.

Genomics applications with *Ruminococcus flavefaciens* FD-1

We are using the utility of BAC libraries for the small genome size of *R. flavefaciens* FD-1. BAC libraries offer a method to perform genetic, physical,

and functional analyses of a bacterial genome without the need for an extensive investment in sequencing or specific methods development. *R. flavefaciens* FD-1, requires only a few hundred clones for equivalent coverage. The BAC library is being screened for the expression of plant cell wall degrading enzymes using: 1.) substrate overlays; 2.) cellulase genes (*celA*, *celB*, *celD* and *celE*) from *R. flavefaciens* FD-1 which have already been cloned and sequenced; and 3.) mRNA from *R. flavefaciens* FD-1 grown with either cellobiose or cellulose as the carbon source. These approaches are being used in order to identify possible clusters of orthologous genes (COGs). Our hypothesis is that many important components of the cellulase system are in COGs. This is based on the limited genomic analysis of cellulase producing organisms (Guglielmi and Beguin, 1998; Ogata et al., 1997) which indicate that while cellulase genes are widely distributed around the chromosome, there is clustering of genes within this scattering. Further support for this hypothesis comes from the complete genome sequence of *Clostridium acetobutylicum*, a closely related organism to *R. flavefaciens* FD-1 (http://www.genomecorp.com/genesequences/clostridium/clospage.html). Inspection of this genome indicates that it contains a complete cellulase complex (cellulosome). One cluster of genes contains nine cellulase ORFs including a CbpA homolog (scaffoldin) spanning a 15.6 kb region of this chromosome. The use of BAC technology to study and identify differentially expressed genes (cellobiose grown versus cellulose grown cells) and activities from a BAC library of *R. flavefaciens* FD-1 will allow us to identify the entire complement of cellulase components.

Genomics applications with *Ruminococcus albus* 8

We have successfully developed differential display RT-PCR (DD RT-PCR) as a viable approach to examine the response of *R. albus* to biochemical and(or) physical cues such as phenylacetic and phenylpropionic acids (PAA/PPA) (Larson and Morrison, 1999). Eighteen putative, differentially expressed sequence tags (dESTs) were identified and 6 of these were confirmed by northern blot analysis to hybridize with differentially expressed transcripts. Genomic DNA fragments encoding two of these dESTs (D4 and D18) have now been isolated and partially characterized (Larson et al., 1999). The D4 dEST was derived from an operon containing 5 genes and all genes possess high sequence similarity to an operon required in *Streptococcus crista* for fimbrial tuft formation (Correia et al., 1997). The D18 dEST was also derived from an operon, containing 4 genes. The first gene encodes a member of the LacI-GalR family of bacterial regulatory proteins, and the second gene encodes a lipoprotein with similarity to a number of carbohydrate binding, transporter proteins from gram-positive bacteria. The final two genes are highly homologous to the *cglF* and *cglG* genes of *Thermoanaerobacter brockii*, which are presumed to encode the integral membrane proteins of an ABC transport system (Breves et al., 1997). It is unlikely that knowledge of these systems would have been derived via screening genomic libraries in *E. coli*.

Recent advances in the development of cloning vectors and high throughput sequencing technologies can revolutionize our approaches to better understand rumen bacterial physiology. In other fields of microbiology, the state-of-the-art is represented by having DNA sequence data for the entire genome. If rumen microbiology is to remain a viable area of research in the future, then its community of scientists must take the steps necessary to ensure that genome sequence data is obtained for as many ruminal microorganisms as possible, in addition to exploiting the wealth of information obtained from other members of the prokaryote world.

THE NORTH AMERICAN CONSORTIUM FOR THE GENOMICS OF FIBROLYTIC RUMINAL BACTERIA

Recently, efforts in the United States to obtain funding to sequence rumen bacterial genomes have been successful. A Consortium made up of investigators at The Ohio State University (M. Morrison, Lead Institution), the University of Illinois (R. Aminov, R. Mackie, B. White), Cornell University (J. Russell, D. Wilson), the University of Guelph (C. Forsberg), and The Institute for Genomic Research (TIGR; Karen Nelson) has been funded by the United States Department of Agriculture, Initiative for Future Agriculture and Food Systems to sequence to completion the genome of *Fibrobacter succinogenes*. The Consortium will also sequence the genome of *R. albus* 8 to 8X coverage. Additional funds in from the proposal will be used by the cooperating institutions to utilize two enzyme subtractive hybridization, and (or) octamer-based genome scanning (Kim et al., 1999) to examine microbial relatedness and identify novel genes in additional rumen bacteria. We expect this research to result in a genetic blueprint for the predominant microorganisms controlling ruminal fiber degradation. The sequence data will fully enable the use of "post-genomics" technologies and bioinformatics, to elucidate which enzyme(s) or other ecological or physiological process(es) are rate-limiting to fiber degradation, and how this might change relative to dietary composition.

IMPLICATIONS

The results obtained from genomic and proteomic based science can lead to a better understanding of the basic biochemical and physiological mechanisms involved in cellulose hydrolysis not only in ruminal

bacteria but also by fermentative bacteria in any anaerobic ecosystem. Rumen microbiology has reached a crossroads. Its future vitality is largely dependent on how effectively we utilize genomics and proteomics to improve our fundamental understanding of microbial physiology, diversity and ecology, and overcome constraints to ruminal function.

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