BARD

FINAL REPORT

PROJECT NO. US-2783-96

Molecular Based Analysis of Cellulose-Binding Proteins Involved with Adherence to Cellulose by *Ruminococcus albus*

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2000

BARD Final Scientific Report
(Cover Page)

Date of Submission of the report: November 9, 2000

BARD Project Number: US 2783-96
Evaluating Panel: Animal Production

Project Title: Molecular based analysis of cellulose-binding proteins involved with adherence to cellulose by *Ruminococcus albus*.

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Continuation of (Related to) Previous BARD Project:
☐ Yes X No Number:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Cellulosomes, rumen bacteria, fiber degradation, animal productivity

Abbreviations used in the report, in alphabetical order:

CBP cellulose binding protein; **DD-RT-PCR** differential display reverse transcriptase polymerase chain reaction; **PAA** phenylacetic acid; **PPA** phenylpropionic acid.

Budget: IS: $140,000 US: $160,000 Total: $300,000

Signature
Principal Investigator

Signature
Research Authority, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors Only	Israeli Authors only	Total
Refereed (published, in press, accepted)	1	2	1	4
Submitted, in review, in preparation	0	3	1	4
Invited review papers	1			1
Book chapters				0
Books				0
Master theses		1		1
Ph.D. theses		1		1
Abstracts	0	4		4
Not refereed (proceedings, reports, etc.)		1		1

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				0
Issued (allowed)				0
Licensed				0

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	1	0	0	1
Sabbaticals				
Postdoctorates				

Cooperation, briefly explain whether synergistic, complementary or supportive.

During the three years of the project, the research studies in both labs have been largely complementary, with different research approaches employed by each lab to study adhesion. Now that a number of objectives have been accomplished, the Investigators have been able to share these resources and findings between labs, and the investigations can proceed in a synergistic manner, especially with the inclusion of another Israeli partner.

ABSTRACT:

At the beginning of this project, it was clear that *R. albus* adhered tightly to cellulose and its efficient degradation of this polysaccharide was dependent on micromolar concentrations of phenylacetic acid (PAA) and phenylpropionic acid (PPA). The objectives for our research were: i) to identify how many different kinds of cellulose binding proteins are produced by *Ruminococcus albus;* ii) to isolate and clone the genes encoding some of these proteins from the same bacterium; iii) to determine where these various proteins were located and; iv) quantify the relative importance of these proteins in affecting the rate and extent to which the bacterium becomes attached to cellulose. BARD support has facilitated a number of breakthroughs relevant to our fundamental understanding of the adhesion process. **First, *R. albus* possesses multiple mechanisms for adhesion to cellulose.** The P.I.'s laboratory has discovered a novel cellulose-binding protein (CbpC) that belongs to the Pil-protein family, and in particular, the type 4 fimbrial proteins. We have also obtained genetic and biochemical evidence demonstrating that, in addition to CbpC-mediated adhesion, *R. albus* also produces a cellulosome-like complex for adhesion. These breakthroughs resulted from the isolation (in Israel and the US) of spontaneously arising mutants of *R. albus* strains SY3 and 8, which were completely or partially defective in adhesion to cellulose, respectively. While the SY3 mutant strain was incapable of growth with cellulose as the sole carbon source, the strain 8 mutants showed varying abilities to degrade and grow with cellulose. Biochemical and gene cloning experiments have been used in Israel and the US, respectively, to identify what are believed to be key components of a cellulosome. This combination of cellulose adhesion mechanisms has not been identified previously in any bacterium. **Second, differential display, reverse transcription polymerase chain reaction (DD RT-PCR) has been developed for use with *R. albus*.** A major limitation to cellulase research has been the intractability of cellulolytic bacteria to genetic manipulation by techniques such as transposon mutagenesis and gene displacement. The P.I.'s successfully developed DD RT-PCR, which expanded the scope of our research beyond the original objectives of the project, and a subset of the transcripts conditionally expressed in response to PAA and PPA have been identified and characterized. **Third, proteins immunochemically related to the CbpC protein of *R. albus* 8 are present in other *R. albus* strains and *F. intestinalis*.** Western immunoblots have been used to examine additional strains of *R. albus,* as well as other cellulolytic bacteria of ruminant origin, for production of proteins immunochemically related to the CbpC protein. The results of these experiments showed that *R. albus* strains SY3, 7 and B199 all possess a protein of ~25 kDa which cross-reacts with polyclonal anti-CbpC antiserum. Several strains of *Butyrivibrio fibrisolvens, Ruminococcus flavefaciens* strains C-94 and FD-1, and *Fibrobacter succinogenes* S85 produced no proteins that cross-react with the same antiserum. Surprisingly though, *F. intestinalis* strain DR7 does possess a protein(s) of relatively large molecular mass (~200 kDa) that was strongly cross-reactive with the anti-CbpC antiserum. Scientifically, our studies have helped expand the scope of our fundamental understanding of adhesion mechanisms in cellulose-degrading bacteria, and validated the use of RNA-based techniques to examine physiological responses in bacteria that are not amenable to genetic manipulations. Because efficient fiber hydrolysis by many anaerobic bacteria requires both tight adhesion to substrate and a stable cellulosome, we believe our findings are also the first step in providing the resources needed to achieve our long-term goal of increasing fiber digestibility in animals.

2. ACHIEVEMENTS:

2.1 U.S. group:

The achievements from the USA will be presented relative to the statement of objectives presented in the original BARD proposal. Some additional achievements outside the original objectives are also described. At this point, it is also important to explain that the protein referred to as CbpC is a cellulose-binding protein of approximately 20 kDa.

a. CbpC protein homologs are probably present in all *R. albus* strains: We demonstrated that a number of *R. albus* strains not only possess proteins similar in size and antigenicity to CbpC, but that these strains also possess *cbpC* gene homologs (Pegden et al. 1998). These *cbpC* gene homologs have now been cloned from several different strains and they are currently being examined in terms of binding specificity and affinity. A surprising finding from these studies was that *Fibrobacter intestinalis* also produces a very large molecular mass protein, which is strongly reacts with anti-CbpC antibodies. This observation will be pursued further as part of the renewal supported by BARD.

b. CbpC production increases in response to PAA/PPA and growth on cellulose: Both CbpC production and cell adhesion increases in response to PAA/PPA (Pegden et al. 1998). CbpC production is increased approximately 10-fold in response to cellulose, and much of the protein eventually is released from the cell surface and is found in culture fluids.

c. Archael-like genes are present in *R. albus* 8: All but four of the genes in the 10 kilobases surrounding the *cbpC* gene have no significant identity with sequences currently available in the databases. Two share significant identity with sequences found only archaebacterial genomes the other two encode homologs of the SecD and SecF proteins, which are components of the type II secretion system found in many prokaryotes, and are known to be involved with Pil-protein export. We hope to be able to continue this work with BARD support and elucidate the functions associated with these various ORF's.

d. Northern (RNA) blot analysis of *cbpC* expression: *cbpC* mRNA increases dramatically (8- to 10-fold) in response to growth on cellulose, and ribonuclease protection assays have shown that transcription initiation occurs from the same position following growth in either cellobiose or cellulose (Larson, PhD thesis, University of Nebraska). Motifs typically recognized by RNA polymerases which possess a sigma-70 binding protein, as well as additional motifs which could be recognized by DNA-binding protein(s) involved with transcriptional regulation. These results are also part of Marilynn Larson's Ph.D. thesis and will be included in the manuscript submittted to the *Journal of Bacteriology.*

e. Operons encoding functions other than cellulases are conditionally expressed in response to PAA/PPA: Although not part of the original plans for RNA analysis, a major achievement during the BARD-supported research was the successful development and use of DD RT-PCR to examine conditional gene expression in *R. albus.* Dr. Larson has one refereed publication arising from this research (Larson and Morrison 2000) and a second manuscript is being prepared for submission to *Molecular Microbiology.* In brief, a four gene and a six gene operon have been isolated and characterized. Ribonuclease protection mapping confirmed the presence of two promoters in the four gene operon and DNA mobility shift assays performed with *R. albus* cell extracts suggested the presence of *cis*-acting elements upstream of this operon.

f. Adhesion-defective mutants differ in their ability to degrade cellulose: Two main achievements of the US group were the isolation of several independent mutants only *partially* defective in adhesion to cellulose, and demonstrating such mutants differed in degradative potential.

g. Proteins absent in the mutant strains are likely to be part of a cellulosome: The mutant protein profiles were a key factor in identifying proteins implicit to the adhesion process, and the subsequent isolation of a clone containing dockerin modules, consistent with the existence of a cellulosome in *R. albus.* This research is the basis of the Master of

Science thesis successfully prepared and defended by Mr. Sanjay Karnati, and a manuscript has been prepared for submission to *Applied and Environmental Microbiology.*

h. *R. albus* has multiple types of adhesion mechanisms: Mr. Karnati was also able to show that adhesion of the mutant strains to cellulose could be completely blocked by the inclusion of carboxymethylcellulose (CMC) in adhesion assay mixtures. Given that we had shown earlier that CbpC binding is competitively inhibited by CMC (Pegden et al. 1998) we believe a major achievement of our studies to date is to demonstrate the existence of multiple adhesion mechanisms in *R. albus:* one mediated by fimbrial-like structures comprised of the CbpC protein, and a second system analogous to the cellulosome paradigm pioneered by Israeli researchers.

2.2 Israeli group:

The broad objectives of the research proposal were:

a. To identify, isolate, and characterize cellulose binding proteins (CBPs) from *R. albus* SY3.

b. Determine the spatial location of CBPs in SY3, as well as whether and how the expression of CBPs is regulated by nutrient availability.

c. Quantify the relative importance of individual CBPs in terms of adherence to and degradation of cellulose by *R. albus* SY3.

d. Assess the degree of genetic and protein homology among CBPs isolated from different strains of *R. albus* as well as other fibrolytic ruminal bacteria.

All of the above mentioned broad objectives have been achieved as described in three full-length papers either published or submitted for publication. Copies of the manuscripts are attached to the end of the report.

i. J. Miron et al. (1998). An adhesion defective mutant of R. albus SY3 is impaired in its capability to degrade cellulose. Journal of Applied Microbiology, 84: 249-254. ***This paper deals mainly with objectives a and c.***

ii. J. Miron et al. (2000). Distribution of cellulolytic enzymes and cellulose binding proteins among the envelopes of *R. albus* SY3, and their possible role in the process of adhesion to cellulose. Submitted to Journal of Applied Microbiology. ***This paper addresses the scientific objectives a through d, listed above.***

iii. M. Morrison and J. Miron (2000). Adhesion to cellulose by *R. albus:* a combination of cellulosomes and Pil-proteins? FEMS Microbiology Letters, 185: 109-115. ***This review paper summarizes the knowledge obtain throughout the project.***

For much of the project the cooperation between partners was largely complementary, with each lab emphasizing skills and methods routinely used in their respective laboratories. The cooperation between the partners was significantly enhanced by the P.I.'s trip to Israel in July 1999, and the results we have to date provided the impetus behind our decision to write a renewal proposal. We have further strengthened the collaboration by my visit to Israel through our inclusion of Dr. E.A. Bayer (Weizmann Institute of Science) as a co-P.I. on the renewal proposal. We thus expect that rapid progress will be made and the sharing of results among us will significantly strengthen the synergistic cooperation in the future.

The following is a list of publications either published, submitted, or in the final stages of preparation, which include results arising from this BARD project. The publications themselves are included as requested. Most of the results included in these publications are fundamental in nature, rather than detailed studies demonstrating either proof of concept (i.e. improved fiber digestibility) or economic implications. However, as mentioned in our original application, we believed studies of this nature were necessary, the first step towards understanding the mechanisms of bacterial adhesion to cellulose in the rumen environment. Furthermore, these studies have provided new insights into the adhesion of

bacteria to plant surfaces, and call attention to the likely existence of genetically analogous adhesion determinants in both pathogenic and non-pathogenic bacteria.

3. Publications (peer reviewed):

1. Miron, J., Morag, E., Bayer, E.A., Lamed, R. and Ben-Ghedalia, D. (1998) An adhesion-defective mutant of *Ruminococcus albus* SY3 is impaired in its ability to degrade cellulose. J. Appl. Microbiol. 84: 249-254.

2. Pegden R.S., Larson, M.A., Grant, R.J. and Morrison, M. (1998) Adherence of the gram-positive bacterium *Ruminococcus albus* to cellulose and identification of a novel form of cellulose-binding protein which belongs to the Pil family of proteins. J. Bacteriol. 180: 5921-5927.

3. Larson M.A., and Morrison, M. (1999) Application of the differential-display RT-PCR technique to examine conditional gene expression in *Ruminococcus albus.* Proc. 8th Int. Symp. on Microb. Ecol.

4. Morrison, M. and Miron, J. (1999) Adhesioin to cellulose by *Ruminococcus albus:* the cellulosome paradigm, and a case for comparative pathobiology? FEMS Microbiol. Letts. 185: 109-115

Manuscripts submitted for publication or in preparation:

5. Miron J., Jacobovitch, J., Bayer, E.A., Lamed, R., and Ben-Ghedalia D. Distribution of cellulolytic enzymes and cellulose binding proteins among the envelopes of *R. albus* SY3, and their possible role in the process of adhesion to cellulose. Submitted to Journal of Applied Microbiology.

6. Larson, M.A. and Morrison, M. Differential Expression of the *Ruminococcus albus parABCD* Operon Encoding a LacI-GalR Regulatory Gene Product and Binding

Protein-Dependent Transport System in Response to Phenyl-substituted Fatty Acids. In preparation for *Molecular Microbiology.*

7. Larson, M.A. and Morrison, M. Transcriptional Up-regulation of the Cellulose-binding Protein CbpC Gene in *Ruminococcus albus* in Response to Cellulose, and Expression of the Adjacent Downstream Type II Secretion Components. In preparation for *Journal of Bacteriology.*

Differential Expression of the *Ruminococcus albus parABCD* Operon Encoding a LacI-GalR Regulatory Gene Product and Binding Protein-Dependent Transport System in Response to Phenyl-substituted Fatty Acids

by

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Running Title: Differential expression in *Ruminococcus albus*

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This manuscript has been assigned Journal Series No. ???, Agricultural Research Division, University of Nebraska.

ABSTRACT

In *Ruminococcus albus* the differentially expressed sequence tag (dEST) D18, obtained using differential display, hybridized with a 4.7 and 3.6 kb transcript. The abundance of these two transcripts was maximal when phenylacetate (PAA) and phenylpropionate (PPA) or ruminal fluid were included in the growth medium. The genomic DNA corresponding to and flanking dEST D18 revealed an operon which encodes four genes, and was referred to as the *parABCD* (phenyl-substituted acid responsive) operon. The *parA* gene encodes a protein with notable sequence similarity to members of the LacI-GalR family of regulatory proteins and *parBCD* appear to encode the structural components of a binding protein-dependent uptake system. Northern blot analyses with probes specific to each ORF revealed that the 4.7 kb polycistonic transcript contained all four ORFs, whereas the 3.6 kb transcript did not encode the *lacI-galR* regulatory gene (*parA*). A 2.7 and 1.6 kb transcript was also revealed which encoded ParAB and ParB, respectively. The absence of *parA* in the 3.6 and 1.6 kb transcripts suggests a possible autoregulatory role for ParA. Moreover, periodic transcriptional read-through at the transcriptional termination signal after *parB* suggests an antitermination mechanism. Ribonuclease protection mapping of the 5' termini of these transcripts confirmed the presence of two promoters, one positioned upstream of *parA* (P1) and the other upstream of *parB* (P2). These two transcriptional start sites were the same, regardless of whether the available carbon source was cellobiose or cellulose, with or without the provision of ruminal fluid or PAA/PPA. The four *par* transcripts derived from either the P1 or P2 promoter were all expressed in greater abundance, with the inclusion of ruminal fluid or PAA/PPA. DNA mobility shift assays

performed with *R. albus* cell extracts suggested the presence of *cis*-acting elements affecting P1-directed transcription.

INTRODUCTION

Several physiochemical factors are known to affect microbial adhesion to and degradation of cellulose (Minato *et al.*, 1993; Pell and Schofield, 1993). For example, proficient growth of *Ruminococcus albus,* one of the three predominant ruminal cellulolytic bacteria, with cellulose as the sole carbon and energy source is dependent on the inclusion of ruminal fluid in the growth medium (Wood *et al.* 1982). Stack, Hungate, and co-workers (1982, 1983, 1984) determined that the principal stimulatory components in ruminal fluid were phenylacetic acid (PAA) and phenylpropionic acid (PPA). Micromolar concentrations of PAA and PPA greatly stimulate cellulase enzyme production by *R. albus*, as well as the number of cells adherent to cellulose and cellulose digestion kinetics. *R. albus* cell morphology is also altered: both vesicular and fimbrial structures are produced, there is an increase in the thickness of the glycocalyx capsule surrounding the cell wall, and cellulases remain associated with the bacterial capsule as part of large molecular mass protein complexes. Phenyl-substituted acids are commonly found in microbial habitats rich in plant biomass, as a result of the biotransformations of lignin, caffeic acids, flavanoids, aromatic amino acids, and some of their metabolites (Harborne, 1980; Colberg, 1982; Burlingame and Chapman, 1983). However, no other known cellulolytic bacteria have been found to respond in this manner to such compounds. Moreover, the gene products and regulation of those genes responsible for this PAA/PPA-responsive phenotype of *R. albus* have

Transcriptional Up-regulation of the *Cellulose-binding Protein CbpC* Gene in *Ruminococcus albus* in Response to Cellulose and Expression of the Adjacent Downstream Type II Secretion Components

by

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Running Title: Cellulose responsive *Ruminococcus albus cbpC*

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This manuscript has been assigned journal series no. ???, Agricultural Research Division, University of Nebraska.

ABSTRACT

The 17,688 Da cellulose-binding protein C (CbpC) was previously isolated from *Ruminococcus albus* 8 by a cellulose-binding affinity assay and the corresponding gene was identified by a combination of reverse genetics and genomic walking procedures. Northern blot analysis showed that the *cbpC* transcript is present in cellulose and cellobiose-grown cells. Moreover, the abundance of the *cbpC* transcript is 7.6-fold and 4.2-fold greater in the cellulose-grown cells relative to the cellobiose-grown cells with the provision of ruminal fluid or phenylacetate (PAA) and phenylpropionate (PPA), respectively. This apparent increase in the *cbpC* transcript abundance concurs with the results obtained in adhesion assays and western blot analyses using anti-CbpC antibodies. Downstream of the *cbpC* gene is an open reading frame (ORF), designated *pilX*. Similar to CbpC, PilX possesses an amino-terminal motif characteristic of the Pil family of proteins, which are usually involved with the formation of type 4 fimbriae and other surface associated protein complexes. Adjacent and directly downstream of *pilX* are two transcriptionally-linked ORFs with notable amino acid identity to the SecD and SecF integral membrane proteins of type II secretion systems. Unlike the 0.7 kb *cbpC* transcript, the 2.5 kb "*secDF*" and 0.57 kb *pilX* transcripts are not up-regulated in response to the presence of cellulose or PAA/PPA. Together these results suggest that the downstream gene products may play a role in the proper assembly and secretion of a CbpC-structure/complex and that CbpC is directly involved in adhesion of *R. albus* to cellulose.