

## FINAL REPORT for DOE GRANT DEFG02-04ER15553

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**Project:** Structure, Function and Regulation of the *Clostridium cellulovorans* Cellulosome

**Principal Investigator:** Roy H. Doi

**Address:** Section of Molecular & Cellular Biology  
University of California  
One Shields Avenue  
Davis, CA 95616  
Tel: (530) 752-3191  
Fax: (530) 752-3085  
E-mail: rhdoi@ucdavis.edu

**DOE Project Personnel:** Dr. Satoshi Matsuoka  
Dr. Akihiko Kosugi  
Dr. Takamitsu Arai  
Dr. Hyunju Cha  
Dr. Jaeho Cha  
Dr. Sung Ok Han  
Dr. Roger Koukiekolo  
Ms. Helen Chan

### Progress Report:

Our major goal for this project (2004-2008) was to obtain an understanding of the structure, function, and regulation of the *Clostridium cellulovorans* cellulosomes. Our specific goals were to select genes for cellulosomal and non-cellulosomal enzymes and characterize their products, to study the synergistic action between cellulosomal and non-cellulosomal enzymes, to study the composition of cellulosomes when cells were grown with different carbon sources, continue our studies on the scaffolding protein and examine heterologous expression of cellulosomal genes in *Bacillus subtilis*. We fulfilled the specific goals of our proposal. The superscripts refer to the papers listed below.

1. In our continuing search for cellulase and hemicellulase genes, we were able to clone a non-cellulosomal gene, *engO*, a family 9 endoglucanase<sup>3</sup> and *bglA*, a  $\beta$ -glucan glucohydrolase<sup>6</sup>. It has become apparent from our studies that non-cellulosomal enzymes work synergistically with the cellulosome. A question that has puzzled us was the fact that there were so many family 9 enzymes. An analysis of their properties revealed that the different family 9 enzymes had different substrate specificities and produced different products<sup>5</sup>. Thus depending on the substrates that are available and the conditions in which the cell finds itself, different family 9 enzymes will be produced. This will also result in a heterologous population of cellulosomes with different enzymatic specificities.

2. In our studies on synergism, we found that a combination of hemicellulases and cellulases was very effective in the degradation of corn fiber<sup>2</sup> and sugar cane bagasse<sup>16</sup>. Thus, it is apparent that the cellulosome is effective in degrading natural plant cell wall materials by the presence of both cellulases and hemicellulases. Since there may be more than 50 different cellulase and hemicellulases, the heterogeneous mixture of cellulosomes again makes them capable of degrading a variety of plant cell wall materials.
3. The expression of cellulosomal genes was observed at the mRNA level in previous studies. A further analysis of the cellulosomal genes under different conditions of growth revealed that depending on the carbon source, different patterns of gene expression were observed. The expression of the major cellulosomal genes, *cbpA*, *engE*, *exgS* and *xynA*, were observed with all carbon sources. However, the expression of other cellulases and hemicellulases varied significantly when the carbon source was cellulose, xylan or pectin<sup>1</sup>. This suggests a possible way to optimize the composition of cellulosomes to degrade specific substrates.
4. In a study of the properties of the cellulose binding domain (CBD) of the cellulosomal scaffolding protein (CbpA), it was found that a linear array of amino acids acts as the binding site of CBD to the substrate. Mutations of these amino acids disrupted the binding ability of CBD to cellulose<sup>4</sup>. In the analysis of HbpA, a member of the cellulosomal cluster of genes, it was found to have synergistic effects with cellulosomal cellulases<sup>12</sup>. In a study of cohesin-dockerin interactions, it was observed that higher copy numbers of cohesins in mini-CbpA facilitated a greater binding of cellulosomal enzymes. This suggests that the 9 copies of cohesins in CbpA make it a very effective scaffolding protein and enhances its activity<sup>13</sup>. A systematic study of physical and chemical conditions of binding of mini-CbpA to a crystalline substrate revealed the optimum conditions for these interactions<sup>17</sup>. These studies indicate however that the interaction of the scaffolding protein, CbpA, with the cellulosomal enzymes and the substrate is complex and requires further studies.
5. In order to enhance the production of cellulosomes, we have attempted to convert an aerobic bacterium to a cellulosome producer. In a previous study, we had shown that *Bacillus subtilis* could express a minicellulosome. In an extension of these studies we have found a novel phenomenon which we have called "intercellular complementation"<sup>8</sup>. We expressed a mini-CbpA in one *B. subtilis* strain and a cellulosomal endoglucanase in another strain. By co-culturing the two strains, we obtained a minicellulosome containing the endoglucanase. Thus each strain produced and secreted their protein into the culture and joined to form a mini-cellulosome. By introducing more *C. cellulovorans* cellulase and hemicellulase genes into *B. subtilis*, we should be able to construct strains of *B. subtilis* that can produce a mixture of mini-cellulosomes. Since aerobic bacteria are easier to handle and are more productive than anaerobic bacteria, the conversion of an aerobic bacterium to a cellulosome producer may facilitate the production of high levels of cellulosomes.
6. Several invited reviews were written to summarize our current understanding of the structure and function of the cellulosome from mesophilic microorganisms<sup>7,9,10,11,14,15</sup>.

With the growing interest in producing Biofuels from plant materials, these reviews should be useful for scientists from many fields.

7. We have had very successful collaborations with Drs. Yukawa and Inui of the Research Institute of Innovative Technology for the Earth (RITE), Kyoto, Japan<sup>1,2,3,5,8,12,13</sup> and with Dr. Brett Pletschke of the Department of Biochemistry, Microbiology & Biotechnology, Rhodes University, Grahamstown, South Africa<sup>16,17</sup>.

**Summary:** Our studies during the past four years on the cellulosome of *Clostridium cellulovorans* have provided significant understanding of the function of the scaffolding protein, the regulation of expression of cellulosomal genes, the effect of carbon source on cellulase and hemicellulase gene expression, the heterogeneity of the population of cellulosomes and heterologous expression of cellulosomeal genes in *Bacillus subtilis*.

### **Presentations of our work on cellulosomes**

1. American Society for Microbiology, New Orleans, LA (May 2004)

Han, S.-O. Yukawa, H. Inui, M. Doi, R.H.	Effect of carbon source on the cellulosomal (sub-) population of <i>Clostridium cellulovorans</i>
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Koukiekolo, R. Yukawa, H. enzymes Doi, R.H.	Characterization of the <i>Clostridium cellulovorans</i> cellulosome and its interaction with non-cellulosomal Inui, M.
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Cho, H.-Y. Yukawa, H. Inui, M. Doi, R.H.	Construction and production of designer cellulosome of <i>Clostridium cellulovorans</i> in <i>Bacillus subtilis</i> .
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Arai, T. Kosugi, A. Chan, H. Koukiekolo, R. Doi, R.H.	Properties and mutation analysis of the EngH, an enzymatic subunit of the <i>Clostridium cellulovorans</i> cellulosome.
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2. Japan Society for Bioscience, Biotechnology, and Agrochemistry, Tokyo, Japan (Nov. 13, 2004). **Invited Speaker.**

Doi, R.H.	Microbial Biotechnology and Future Applications.
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3. International Workshop on Biorefinery and Bioenergy, Kyoto, Japan (Feb. 9-10, 2005). **Invited speaker.**

Doi, R.H. Potential of Innovative Biomass Saccharification Technologies: Utilization of Cellulosome Functions

4. American Society for Microbiology, Atlanta, GA (June 2005)

Arai, T. Properties of cellulosomal family 9 cellulases  
Kosugi, A. from *Clostridium cellulovorans*.  
Chan, H.  
Koukiekolo, R.  
Doi, R.H.

5. Gordon Research Conference on Cellulases, Andover, New Hampshire (August 2005). **Invited Speaker.**

Doi, R.H. *Clostridium cellulovorans* Cellulosomes: Variation in Dockerin-Cohesin Specificity and Cellulosome Assembly Via Intercellular Complementation

Arai, T. A comparison of family 9 cellulases from  
Kosugi, A. *Clostridium cellulovorans*.  
Chan, H.  
Koukiekolo, R.  
Doi, R.H.

Kosugi, A. A noncellulosomal  $\beta$ -glucan glucohydrolase, BglA,  
Arai, T. cooperates to degrade cello-oligosaccharides  
Doi, R.H. produced from cellulose by *Clostridium cellulovorans*.

6. International Symposium on Extremophiles and their Applications, Tokyo, Japan (November 2005). **Invited Speaker.**

Doi, R.H. Plant Cell Wall Degradation by Intercellular Complementation or Cell-Cell Interaction”.

7. Microbial Production of Energy, The American Academy of Microbiology, San Francisco, CA. (March 2006). **Invited Participant.**

8. American Society for Microbiology, Orlando, FL (May 2006)

Matsuoka, S. Expression of cellulosomal genes from *Clostridium*  
Arai, T. *cellulovorans* in *Bacillus subtilis* WB800 and  
Doi, R.H. secretion of their products. ASM Abstr. P. 104.

9. Incredible Anaerobes: From Physiology to Genomics to Fuels, U of Georgia, Athens, GA (March 2007). **Invited Lecturer.**

Doi, R.H.                      How mesophilic *Clostridium cellulovorans* degrades plant cell walls.

10. American Society for Microbiology, Toronto, Canada (May 2007). **Invited Symposium Speaker.**

Doi, R.H.                      “Turning plant cell walls into sugars with *Clostridium cellulovorans* cellulosomes”. Symposium on Developments in the Conversion of Complex Polysaccharides into Renewable Energy.

Matsuoka, S.  
Doi, R.H.                      Multiple expression of cellulase and hemicellulase enzymes from *Clostridium cellulovorans* in *Bacillus subtilis* WB800.

Cha, J.  
Matsuoka, S.  
Chan, H.  
Doi, R.H.                      Effect of multiple copies of cohesins on cellulase and hemicellulase activities of *Clostridium cellulovorans* minicellulosomes.

11. Gordon Research Conference, Cellulases and Cellulosomes, Andover, New Hampshire (Jul-Aug, 2007). **Invited Discussion Leader.**

Matsuoka, S.    Construction of minicellulosome from *Clostridium*  
Doi, R.H.                      *cellulovorans* in *Bacillus subtilis* WB800.

12. South African Society for Microbiology, Grahamstown, South Africa (Jan 20-25, 2008). **Invited plenary speaker.**

Doi, R.H.                      How *clostridium cellulovorans* cellulosomes (cellulases) convert plant biomass to sugars on the way to Biofuels.

13. Seminar at Molecular Cellular & Developmental Biology/Ohio State Biochemistry, Columbus, Ohio (Mar 4, 2008). **Invited speaker.**

Doi, R.H.                      How *Clostridium cellulovorans* Cellulosomes Convert Plant Biomass to Sugars on the Way to Biofuels

14. NAIST Microbiology Workshop, Nara, Japan (Mar 21-25, 2008). **Invited speaker.**

Doi, R.H.                      Structure and function of *Clostridium cellulovorans*

cellulosome

15. Seminar at Dept. of Biotechnology, Korea University, Seoul, Korea (Apr 24, 2008).

Doi, R.H.                      How *Clostridium cellulovorans* Cellulosomes Convert  
Plant Biomass to Sugars on the Way to Biofuels

16. White Biotechnology Symposium, Jeju Island, Korea (April 25, 2008).

**Invited speaker.**

Doi, R.H.                      Conversion of Plant Cell Walls to Sugars by  
*Clostridium cellulovorans* Cellulosomes

## List of publications resulting from this project (2004-2009):

1. **Han, S-O., Yukawa, H., Inui, M. and Doi, R.H.** Effect of carbon source on the cellulosomal subpopulations of *Clostridium cellulovorans*. *Microbiology* **151**:1491-1497 (2005).
2. **Koukiekolo, R., Cho, H-Y., Kosugi, A., Inui, M., Yukawa, H. and Doi, R.H.** Degradation of corn fiber by *Clostridium cellulovorans* cellulases and hemicellulases and contribution of scaffolding protein, CbpA. *Appl. Environ. Microbiol.* **71**:3504-3511 (2005).
3. **Han, S.-O., Yukawa, H., Inui, M. and Doi, R.H.** Molecular cloning, transcriptional and expression analysis of engO, encoding a non cellulosomal family 9 enzyme from *Clostridium cellulovorans*. *J. Bacteriol.* **187**:4884-4889 (2005).
4. **Murashima, K., Kosugi, A., and Doi, R.H.** Site-directed mutagenesis and expression of the soluble form of the family IIIa cellulose binding domain from the cellulosomal scaffolding protein of *Clostridium cellulovorans*. *J. Bacteriol.* **187**: 7146-7149 (2005).
5. **Arai, T., Kosugi, A., Chan, H., Koukiekolo, R., Yukawa, H., Inui, M. and Doi, R.H.** Properties of cellulosomal family 9 cellulases from *Clostridium cellulovorans*. *Appl. Microbiol Biotechnol.* **71**: 654-660 (2006).
6. **Kosugi, A., Arai, T. and Doi, R.H.** Degradation of cellulosome-produced cello-oligosaccharides by an extracellular non-cellulosomal b-glucan glucohydrolase, BglA, from *Clostridium cellulovorans*. *Biochem. Biophys. Res. Commun.* **349**:20-23 (2006).
7. **Doi, R.H.** The *Clostridium cellulovorans* Cellulosome, pp. 153-168. In V. Uversky and I.A. Kataeva (Eds.), *Cellulosome*, Nova Science Publishers, Inc., Hauppauge, NY (2006).
8. **Arai, T., Matsuoka, S., Cho, H.-Y., Yukawa, H., Inui, M., Wong, S.-L., and Doi, R.H.** Synthesis of *Clostridium cellulovorans* minicellulosomes by inter-cellular complementation. *Proc. Natl. Acad. Sci. USA* **104**:1456-1460 (2007).
9. **Norris, V., den Blaauwen, T., Cabin-Flaman, A., Doi, R.H., Harshey, R., Janniere, L., Jimenez-sanchez, A., Jin, D. J., Levin, P.A., Mileykovskaya, E., Minsky, A., Pugsley, T., Saier, M. Jr., and Skarstad, K.** Functional taxonomy of bacterial hyperstructures. *Microb. Mol. Biol. Rev.* **71**: 230-253 (2007).
10. **Doi, R.H. and Matsuoka, S.** Structure, function and application of cellulolytic

complex, “cellulosome”, of *Clostridium cellulovorans*. Bioscience and Industry 65:121-125 (2007).

11. **Norris, V., den Blaauwen, T., Doi, R.H., Haarshey, R., Janniere, L., Jimenez-Sanchez, A., Jin, D.J., Levin, P.A., Mileykovskaya, E., Minsky, A., Misevic, G., Ripoli, C., Saier, M. Jr., Skarstad, K. and Thellier, M.** Towards a hyperstructure taxonomy. *Ann. Rev. Microbiol.* **61**:309-329 (2007).
12. **Matsuoka, S., Yukawa, H., Inui, M. and Doi, R.H.** Synergistic interaction of *Clostridium cellulovorans* cellulosomal cellulases and HbpA. *J. Bacteriol.* **189**: 7190-7194 (2007).
13. **Cha, J., Matsuoka, S., Chan, H., Yukawa, H., Inui, M. and Doi, R.H.** Effect of the multiple copies of cohesins on cellulase and hemicellulase activities of *Clostridium cellulovorans* minicellulosome. *J. Microbiol. Biotech.* **17**:1782-1788 (2007).
14. **Doi, R.H.** Cellulases of mesophilic microorganisms: Cellulosome & non-cellulosome Producers, *Ann. N.Y.Acad. Sci* **1125**:267-279 (2008).
15. **Doi, R.H.** Cellulosomes from mesophilic bacteria. pp. 97-106. In J.D. Wall, C.S. Harwood, and A. Demain (Eds.), *Bioenergy*, American Society of Microbiology, Washington, DC. (2008).
16. **Beukes, N., Chan' H., Doi, R.H. and Pletschke, B.I.** Synergistic associations between *Clostridium cellulovorans* enzymes XynA, ManA and EngE against sugarcane bagasse. *Enzyme Microbial Technol.* **42**:492-498 (2008).
17. **Lavan' L.M., van Dyk' J.S., Chan' H., Doi, R.H. and Pletschke', B.I.** Effect of physical conditions and chemicals on the binding of a mini-CbpA from *Clostridium cellulovorans* to a semi-crystalline cellulose substrate. *Letters Applied Microbiology* **48**:419-425 (2009).