Application of Biotechnology to Rumen Microbiology in Tropical Countries

K.-J. Cheng*, R. G. Clark*, H. Kudo** and C. W. Forsberg***

Abstract

While considerable progress has been made in our understanding of the very complex enzymatic mechanisms of cellulose digestion in ruminants, the only practical manipulations of this process that will be successful in the near future will involve β-glucan digestion by endoglucanase in high quality cereal feeds. To date, we have been able to transfer many components of the rumen cellulolytic microbial genome to recipient bacteria but we have not succeeded in making them functionally cellulolytic, and we have not been able to develop functional cell-free bacterial enzyme systems that can digest crystalline cellulose. One area in which genetic manipulation appears to be especially promising is in the transfer of the ability to degrade plant toxins by the transfer of specific genes into many different species of the ubiquitous natural flora of domestic animals. Using combinations of simple practical test systems for cellulolytic activity, we have been able to identify a cell-free enzyme system from rumen fungi that is effectively cellulolytic. These tests can readily be used to select microbial isolates which will be used to produce inocula for manipulation of the cellulolytic activity of ruminants on low-quality feeds. When the genetic determinants of enhanced cellulolytic activity have been identified, we will explore the feasibility of their use in developing transgenic ruminant animals producing salivary cellulases.

Introduction

Because the digestion of cellulose by rumen bacteria lies at the heart of a very important area of animal production, this process has been advanced, by many scientists, as a particularly inviting target for manipulation by the exciting new techniques of molecular genetics. The development of strategies for genetic manipulation of rumen microorganisms, and the progress that has been made in this area, have been reviewed by a number of authors (Flores, 1989 ; Gregg *et al.*, 1989 ; Forsberg and Cheng, 1991 ; Malburg *et al.*, 1991), and have been the subject of a recent conference proceedings edited by Akin, Ljungdahl, Wilson and Harris (1990). In spite of this surge of interest, very little practical progress has been made toward the general objective of improvement of feed efficiency by genetic manipulation.

This initial disappointment has come about because of a surprising heterogeneity of cellulosic substrates, the complexity of the microbial enzyme systems involved in the digestion, and the large number of ecological factors which impinge on the overall process of cellulose digestion by ruminants. Detailed analysis of the endoglucanases produced by *Fibrobacter succinogenes* alone has revealed at least 13 different enzymes encoded by 13 different genes (Malburg and Forsberg, 1991). *Ruminococcus albus* and *Ruminococcus*

* Agriculture Canada Research Station, Lethbridge, Alberta, Canada T1J 4B1.
** Eco-physiology Division, Tropical Agriculture Research Centre, Tsukuba, 305 Japan.
*** Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

flavefaciens also produce a bewildering array of cellulolytic enzymes (Howard and White, 1988 ; Huang *et al.*, 1989). Because of this enzymatic complexity, we should not be surprised that we have not yet been able to identify and transfer a sufficient number of genes to simply empower recipient bacteria to digest crystalline cellulose. At the present rate of progress, it will be many years before even rudimentary cellulose digestion can be transferred to wild native rumen populations.

Another serious problem encountered in the genetic manipulation of rumen populations is the basic ecological principle that genetic capacity must be delivered in a species that can survive and thrive in the rumen ecosystem on a wide variety of feeding regimens. The rumen comprises a very stable microbial ecosystem and extraneous microorganisms usually simply pass through this organ system without becoming established as part of the autochthonous population. For these reasons, it is useful to pause at the end of this decade of genetic studies of rumen microbes to reconsider our basic strategies for the practical manipulation of this most important component of feed resource utilization. *In vitro* digestion of crystalline cellulose has been achieved by cell-free enzymes produced by rumen fungi (Wood *et al.*, 1986 ; H. D. Bae, H. Zhu, C. W. Forsberg, and K.-J. Cheng, unpublished data). We have, in one instance, been able to transfer the endoglucanase and xylanase genes of one species of rumen fungus (*Neocallimastix patriciarum*) to *E. coli* (Y. Hu, C. W. Forsberg and K.-J. Cheng, unpublished data). Some measure of cellulose digestion can be effected by cell-free fungal extracts, which implies that the fungal cellulases may be less complex than the corresponding bacterial enzyme systems. We have developed therefore, a collection of rumen fungal cultures isolated from several countries. We are currently assessing the minimum number of genetic elements that must be transferred in order to enable the recipient organism to digest crystalline cellulose. We are also exploring the rumen system, in terms of autochthonous microbial species, to select a recipient organism that is stable and ubiquitous in all feed regimens and sufficiently adherent to either tissues or digesta to be effectively retained in this organ system.

Development of a strategy for practical genetic manipulation

The logical sequence for the development of a practical means of genetic manipulation to enhance cellulose digestion must begin with the isolation of appropriate genes from the most active cellulolytic microorganisms. These optimal genes must then be effectively transferred into cellular "vehicles" that will allow their integration and effective functioning in the natural rumen system.

Identification of the most actively cellulolytic microorganisms

We have started an intensive program to obtain samples of rumen fluid from animals which are able to survive on very poor quality feeds. These include Malaysian cattle and water buffalo maintained on palm press fiber, goats in Ghana maintained on plantain and cassava peels, and wild Canadian herbivores (moose, wapiti, wood buffalo) maintained on native forage. These rumen samples are enriched with crystalline cellulose and the predominant cellulolytic bacteria and fungi are isolated. Their individual rates of cellulose and hemicellulose digestion are assessed by clearance zone size in cellulose plates and by assays of cellulase and hemicellulase activity. This survey of microorganisms from Southeast Asia, Africa, and North America has already provided us with an extensive collection of highly cellulolytic rumen bacteria and fungi. In parallel with our program of genetic engineering, we will introduce the most actively cellulolytic of these microorganisms into newborn calves and lambs to assess their ability to colonize the digestive systems of these animals and improve their digestion of low-quality feeds. In the event that natural strains of bacteria or

fungi from exotic sources can be found to improve cellulose digestion in recipient animals, many regulatory and environmental problems inherent in the use of genetically engineered strains will be avoided.

Characterization of minimal effective cellulolytic enzyme systems

While whole cells of cellulolytic rumen bacteria (*F. succinogenes, R. albus* and *R. flavefaciens*) are readily able to attach to crystalline cellulose and digest this substrate, cell-free extracts of all three organisms are unable to carry out this process. This indicates that the cellulolytic activities of these organisms are complex and may involve some element of physical organization. Each of these species is thought to produce an excess of 8 separate endoglucanases. These enzymes are encoded by genes that are believed to be widely separated on the bacterial chromosome and that must, therefore, be assumed to be expressed in an uncoordinated pattern. We have documented previously the enzymes involved in polysaccharide digestion, and some of the genes that encode these proteins (Sipat *et al.*, 1987 ; Gong *et al.*, 1989 ; McGavin *et al.*, 1989, 1990 ; Huang and Forsberg, 1990 ; Forsberg and Cheng, 1991 ; Hu *et al.*, 1991 ; Malburg *et al.*, 1991). To date, several of these cellulase and hemicellulase genes have been transferred to *E. coli* and to certain rumen bacteria without concomitant transfer to these recipients of the ability to digest crystalline cellulose. These recipient organisms do, however, acquire the ability to digest certain artificial substrates with structures similar to cellulose (e.g., carboxymethyl cellulose, p-nitrophenyl-β-D-cellobioside, p-nitrophenyl-β-D-glucoside).

Rumen fungi have recently been shown to produce enzymes that can digest crystalline cellulose in cell-free preparations (Wood *et al.*, 1986). We have been able to transfer endoglucanase and xylanase genes from one of these fungi (*Neocallimastix patriciarum*) to *E. coli* and we are currently characterizing the enzymes produced by the recipient strain. Even the fungal enzyme systems that digest crystalline cellulose are exhibiting a measure of complexity, thus it is now clear that we must determine the minimum genetic information that will allow a recipient organism to adhere to this solid substrate and effect its digestion. When we have this information, the true magnitude of the task of transferring effective cellulolytic capacity between organisms can be realistically assessed. For this reason, Dr. Forsberg's group will intensify its examination of the activity of purified enzymes in the digestion of cellulose, in order to define the minimum enzymatic requirements necessary for this process.

Exploitation of our current capacity for genetic manipulation

While the enzymatic "machinery" necessary for the digestion of crystalline cellulose is obviously very complicated, individual enzymes within this complex are clearly defined and their genetic control is well understood. For example, the endoglucanase (gene (Cel-3) product EG3) produced by *F. succinogenes* has high digestive activity against the β-glucan component of cereal grains (McGavin *et al.*, 1989). This enzyme activity is well developed in ruminants but it is often much less developed in the microbial population of monogastric animals (Jonsson and Hemmingsson, 1991). A new program has been initiated to transfer the endoglucanase gene from *F. succinogenes* to *Lactobacillus acidophilus* in order to produce an organism that will attach to the intestinal wall of monogastric animals (pig, chicken, human) and produce endoglucanase to digest the viscous β-glucans of barley cell walls. Baik and Pack (1990) have been successful in transferring an endoglucanase gene from *Bacillus subtilis* into *L. acidophilus*. The resultant colonization of intestinal epithelial tissue, and the more complete digestion of viscous β-glucans, will prevent digestive disturbances caused by pathogenic bacteria and promote better feed utilization.

Other polysaccharide-degrading enzymes (e.g., hemicellulase) are in the process of being characterized and their encoding genes sequenced to enable transfer of these genes from *F. succinogenes* or rumen fungi to *Lactobacillus plantarum* so that this recipient organism can be used to improve the preservation of silages prepared from grasses and legumes high in fiber and low in water soluble carbohydrates, by enabling the organism to convert glucans and xylans to lactic acid. Our colleague, Collins Thompson, at the University of Guelph, is planning the transfer of either the Cel-3 gene (McGavin *et al.*, 1989) or a *B. subtilis* gene. The recipient organism will likely be *L. acidophilus*, since *L. plantarum* has already been successfully genetically manipulated (Scheirlink *et al.*, 1990).

As scientists continue to characterize the numerous enzymes that make up the complex cellulose digestion machinery of rumen bacteria and fungi, we will discover a remarkable variety of specific catalytic activities. These enzymatic activities, and the genes that encode these proteins, may have a remarkable potential for the production of a whole battery of polysaccharide-modifying enzymes of considerable economic importance in industrial biotransformations.

Long-term aims of genetic manipulation

The aspects of ruminant digestion that have been suggested as the most important targets of genetic manipulation are : 1) polysaccharide digestion, 2) detoxification of plant toxins (Gregg and Sharpe, 1991), and 3) microbial metabolism of protein. This first area has received the most attention to date and the piecing together of the individual enzymatic components in this potentially very complex process is just beginning (Whitehead and Hespell, 1990 ; Whitehead *et al.*, 1991). The most important problem remaining in the exploitation of genetic manipulation in this critical area is the definition of the enzymatic and non-enzymatic components that limit the digestion of lignocellulose, and the development of suitable genetic engineering methods. In the second area, scientists in several countries have identified bacteria with the capacity to degrade specific plant toxins and have transferred this capacity between animals (Cheng *et al.*, 1985 ; Jones and Megarrity, 1986 ; Kudo *et al.*, 1990). However, these detoxifying strains of bacteria may not always become established in the autochthonous populations of recipient animals under certain dietary conditions, therefore Australian scientists have undertaken to transfer the genes controlling detoxification to ubiquitous native rumen organisms (Gregg and Sharpe, 1991). The third area, microbial protein metabolism, has perhaps the greatest potential to improve the efficiency of animal production but we lack detailed information on microbial protein metabolism in different diets. As with the other areas, manipulation of ruminal protein metabolism requires that any modified organisms introduced into the rumen become integrated into the natural resident microbial populations of this organ system.

Ecological principles predict that extraneous organisms will simply pass through a stable ecosystem without integration into its resident autochthonous populations. This is demonstrably true of the rumen (Adams *et al.*, 1966) and we find that effective manipulation of the system by inoculation is only possible when the system is not yet colonized, as in newborn animals, or when the system is profoundly disturbed (Cheng, K.-J., unpublished data). Thus, the only ecologically sound means of manipulating rumen processes is by the early introduction of a native species that has received the genetic material in question. The biological vehicle for this transfer of metabolic activity must be able to adhere firmly either to feed material or to the tissues of the digestive tract, and to integrate completely into the normal autochthonous population of the organ system.

For these reasons, we have chosen *Butyrivibrio fibrisolvens* as the recipient organism for cellulolytic genes because it is a ubiquitous component of the autochthonous rumen microbial population, on all known feed regimens, and because it attaches avidly to feed particles

(Cheng *et al.*, 1983-84). In this way, a valuable metabolic activity is added to a native organism that already has many important physiological functions in the rumen. Moreover, *B. fibrisolvens* has a small plasmid that can serve as a useful vector for genetic transfer (Mann *et al.*, 1986).

Clearly, whenever we propose to transfer a specific gene into a natural ecosystem like the rumen, we must use as a "vehicle" a native strain that can adhere, persist, and thrive in its own natural environment. Ultimately, the preferred method of genetic transfer into an animal would be to integrate the transferred gene into the genome of the animal itself (Ebert, 1988) so that the enzyme in question would become constitutive and would be expressed in a suitable organ (e.g., cellulase produced by the salivary gland). A further consideration of this achievement is the potential reduction of methane production. Microbial cellulose digestion is irrevocably linked to methane production, which represents energy lost to animal production. Thus, cellulose digestion by the animal itself may reduce methane generation, thereby increasing the efficiency of animal production as well as minimizing the environmental impact of methane production by ruminants.

References

1) Adams, J. C., Hartman, P. A. and Jacobsen, N. L. (1966) : Longevity of selected exogenous microorganisms in the rumen, Canadian Journal of Microbiology, 12, 363-369.
2) Akin, D. E., Ljungdahl, L. G., Wilson, J. R. and Harris, P. J. (eds.) (1990) : Microbial and plant opportunities to improve lignocellulose utilization by ruminants. Elsevier Science Publishing Co., New York, USA.
3) Baik, B-H. and Pack, M. Y. (1990) : Expression of *Bacillus subtilis* endoglucanase gene in *Lactobacillus acidophilus*. Biotechnology Letters, 12, 919-924.
4) Cheng, K.-J., Stewart, C. S., Dinsdale, D. and Costerton, J. W. (1983-84) : Electron microscopy of bacteria involved in the digestion of plant cell walls. Animal Feed Science and Technology, 10, 93-120.
5) Cheng, K.-J., Phillippe, R. C., Kozub, G. C., Majak, W. and Costerton, J. W. (1985) : Induction of nitrate and nitrite metabolism in bovine rumen fluid and the transfer of this capacity to untreated animals. Canadian Journal of Animal Science, 65, 647-652.
6) Ebert, K. M. (1988) : Gene transfer through embryo microinjection. *In* : Animal Biotechnology. Edited by : Babiuk, L. A. and Phillips, J. P. Pergamon Press, Toronto, Canada, pp. 232-250.
7) Flores, D. A. (1989) : Application of recombinant DNA to rumen microbes for the improvement of low quality feed utilization. Journal of Biotechnology, 10, 95-112.
8) Forsberg, C. W. and Cheng, K.-J. (1991) : Molecular strategies to optimize forage and cereal digeston by ruminants. *In* : Biotechnology and Nutrition. Edited by : Bills, D. and Kung, S. B. Butterworth-Heinemann, Stoneham, Mass. (In press).
9) Gong, J., Lo, R. Y. C. and Forsberg, C. W. (1989) : Molecular cloning and expression in *Escherichia coli* of a cellodextrinase gene from *Bacteroides succinogenes* S85. Applied and Environmental Microbiology, 55, 132-136.
10) Gregg, K., Bauchop, T. and Leng, R. A. (1989) : Genetic engineering of rumen microorganisms. *In* : Biotechnology for Livestock Production. FAO. Plenum Press. N. Y.
11) Gregg, K. and Sharpe, H. (1991) : Enhancement of rumen microbial detoxification by gene transfer. *In* : Physiological Aspects of Digestion and Metabolism in Ruminants. Edited by : Tsuda, T., Sasaki, T. and Kawashima, R. Academic Press, Orlando, U. S. A. pp. 719-736.
12) Howard, G. T. and White, B. A. (1988) : Molecular cloning and expression of cellulase genes from *Ruminococcus albus* 8 in *Escherichia coli* bacteriophage λ. Applied and Environmental Microbiology, 54, 1752-1755.

13) Hu, Y., Smith, D. C., Cheng, K.-J. and Forsberg, C. W. (1991) : Cloning of a xylanase gene from *Fibrobacter succinogenes* 135 and its expression in *Escherichia coli*. Canadian Journal of Microbiology, 37, 554-561.
14) Huang, C. M., Kelly, W. J., Asmundson, R. V. and Yu, P. K. (1989) : Molecular cloning and expression of multiple cellulase genes of *Ruminococcus flavefaciens* strain 186 in *Escherichia coli*. Applied Microbiology and Biotechnology, 31, 265-271.
15) Huang, L. and Forsberg, C. W. (1990) : Cellulose digestion and cellulase regulation and distribution in *Fibrobacter succinogenes* subsp. *succinogenes S85*. Applied and Environmental Microbiology, 56, 1221-1228.
16) Jones, R. J. and Megarrity, R. G. (1986) : Successful transfer of DHP-degrading bacteria from Hawaiian goats to Australian ruminants to overcome the toxicity of *Leucaena*. Australian Veterinary Journal, 63, 259-262.
17) Jonsson, E. and Hemmingsson, S. (1991) : Establishment in the piglet gut of lactobacilli capable of degrading mixed-linked β-glucans. Journal of Applied Bacteriology, 70, 512-516.
18) Kudo, H., Majak, W., Mutalib, A. R., Ho, Y. W. and Cheng, K.-J. (1989) : Microorganisms and degradation of deleterious principles. *In* : Ruminant Physiology and Nutrition in Asia. Edited by : Devendra, C. and Imaizumi, E. Japan Society of Zootechnical Science, Tokyo, Japan. pp. 73-87.
19) Malburg, Jr., L. M. and Forsberg, C. W. (1991) : The multiplicity of endoglucanase genes from *Fibrobacter succinogenes* S85. Proceedings of the Canadian Society of Microbiology, MP5p.
20) Malburg, Jr., L. M., Tamblyn Lee, J. M. and Forsberg, C. W. (1991) : Degradation of cellulose and hemicelluloses by rumen microorganisms. *In* : Microbial Degradation of Natural Products. Edited by : Winkelman, G. VCH Verlagsgesellschaft, Weinham, Federal Republic of Germany. (In press).
21) Mann, S. O., Hazlewood, G. P. and Orpin, C. G. (1986) : Characterization of a cryptic plasmid (pOM1) in *Butyrivibrio fibrisolvens* by restriction endonuclease analysis and its cloning in *Escherichia coli*. Current Microbiology, 13, 17-22.
22) McGavin, M. J., Forsberg, C. W., Crosby, B., Bell, A. W., Dignard, D. and Thomas, D. Y. (1989) : Structure of the *Cel-3* gene from *Fibrobacter succinogenes* S85 and characteristics of the encoded gene product, endoglucanase 3. Journal of Bacteriology, 171, 5587- 5595.
23) McGavin, M. J., Lam, J. and Forsberg, C. W. (1990) : Regulation and distribution of *Fibrobacter succinogenes* S85 endoglucanase. Applied and Environmental Microbiology, 56, 1235-1244.
24) Scheirlink, T., DeMeutter, J., Arnaut, G., Joos, H., Claeyssens, M. and Michiels, F. (1990) : Cloning and expression of cellulase and xylanase genes in *Lactobacillus plantarum*. Applied Microbiology and Biotechnology, 33, 534-541.
25) Sipat, A., Taylor, K. A., Lo, R. Y. C., Forsberg, C. W. and Krell, P. J. (1987) : Molecular cloning of a xylanase gene from *Bacteroides succinogenes* and its expression in *Escherichia coli*. Applied and Environmental Microbiology, 53, 477-481.
26) Whitehead, T. R. and Hespell, R. B. (1990) : Heterologous expression of the *Bacteroides ruminicola* xylanase gene in *Bacteroides fragilis* and *Bacteroides uniformis*. FEMS Microbiological Letters, 66, 61-66.
27) Whitehead, T. R., Cotta, M. A. and Hespell, R. B. (1991) : Introduction of the *Bacteriodes ruminicola* xylanase gene into the *Bacteroides* thetaiotamicron chromosome for production of xylanase activity. Applied and Environmental Microbiology, 57, 277-282.
28) Wood, T. M., Wilson, C. A., McCrae, S. I. and Joblin, K. N. (1986) : A highly active extracellular cellulase from the anaerobic rumen fungus *Neocallimastix frontalis*. FEMS Microbiological Letters, 34, 37-40.

Discussion

Hoffman, D. (ACIAR) : Considering the dynamics and rapid multiplication rate of bacteria, how stable would selection be in this environment?

Answer : Based on a large number of experiments (unpublished data) involving oral inoculation of collected microbial stock cultures into newborn lambs, it appeared that the rumen bacteria selected for inoculation were fairly stable in the rumen despite the dynamics and rapid multiplication rate of the rumen bacteria.