UNIVERSITI PUTRA MALAYSIA

STUDIES ON SOME ANAEROBIC RUMEN BACTERIA WITH SPECIAL REFERENCE TO THEIR ENDOGLUCANASE GENE

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FPV 1996 2

STUDIES ON SOME ANAEROBIC RUMEN BACTERIA WITH SPECIAL REFERENCE TO THEIR ENDOGLUCANASE GENE

By

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Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Veterinary Medicine and Animal Science Universiti Pertanian Malaysia

May, 1996

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere gratitude to the chairman of the supervisory committee, Professor Dr. Ho Yin Wan for her advice, guidance, encouragement and kind understanding throughout this project. Sincere thanks are also extended to the other members of the supervisory committee; Associate Professor Dr. Norhani Abdullah and Dr. Suhaimi Napis for their help and suggestions, and Associate Professor Dr. Yasuo Kobayashi, MIE University, Japan, for the technical and financial support during my stay in Japan. I am also indebted to Professor Dato' Dr. Syed Jalaludin Syed Salim, Vice Chancellor, Universiti Pertanian Malaysia, for his encouragement, advice and help. I am most grateful to Mr. Gator Tekashi, MIE University, Japan, for teaching me the molecular techniques for cloning and electroporation, Dr. Hiroshi Kudo, Japanese Agricultural Research Institute, Japan, for his advice in the maintenance of rumen bacteria and Dr. Ronald Teather for supplying the rumen bacteria, *Butyrivibrio fibrisolvens* OB156.

I must give special thanks to Sieo Chin Chin and Wan Zuhainis for helping me to type this thesis, and all the staff of the Rumen Microbiology Laboratory, especially Khairul, Jivanathan and Sivabalan for their kind help and cooperation. To my fellow labmates Jin Lizhi, Thongsuk Jetana and Darlis, my grateful thanks for their companionship, humour, support and help.

Finally, I must extend my heartfelt thanks and gratitude to my family and Foong Yee for giving their unending support, encouragement and understanding throughout my study in the university .

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LIST OF ABBREVATIONS

bp	-	base pair
CMC	-	Carboxymethylcellulose
CIP	-	Calf intestinal phosphatase
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediaminetetraacetic acid
kbp	-	kilo base pair
LB	-	Luria-Bertani medium
OD	-	Optical density
PAGE	-	Polyacrylamide gel electrophoresis
RNA	-	Ribonucleic acid
SDS	-	Sodium dodecyl sulphate
TAE	-	Tris-acetate buffer
TE	-	Tris-ethylenediaminetetraacetic acid buffer
TER	-	Tris-EDTA-RNAase
U	-	Unit/s
UV	-	Ultraviolet
VFA	-	volatile fatty acids
2YT	-	2x yeast-tryptone medium
kV	-	kilo volt
milisec-		milliseconds

Abstract of the thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfillment of the requirements for the Degree of Master of Science

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MAY 1996

CHAIRMAN: **PROFESSOR HO YIN WAN , PhD.**

FACULTY: **FACULTY OF VETERINARY MEDICINE AND ANIMAL SCIENCE**

Four strains of *Fibrobacter succinogenes* (D1, D3, D5 and D6) and 3 strains of *Ruminococcus flavefaciens* (MD-1, MD-8 and MD-9) were found to be highly fibrolytic, degrading and solubilising Whatman No.1 filter paper strips in Scott and Dehority medium within 16-48 h. Among the 7 bacterial strains, *F. succinogenes* D3 and *R. flavefaciens* MD-1 were two of the most active, being able to solubilise the filter paper strip within 16-24 h, and were used for endoglucanase assay. *R. flavefaciens* MD-1 produced significantly higher endoglucanases activity than *F. succinogenes* D3 at all incubation periods. Analysis of endoglucanase using

polyacrylamide gel electrophoresis (PAGE) revealed that both *F. succinogenes* D3 and *R. flavefaciens* MD-1 possesed 3 endoglucanase isozymes but the bands for each bacterial strain were at different positions The endoglucanase bands for *F. succinogenes* D3 were at Rf 0 40, 0 49 and 0 61, and for *R. flavefaciens* MD-1 at Rf 0 66, 0 74 and 0 84 A 5 1 kbp *Bam*HI gene fragment expressing endoglucanase activity was cloned from *R. flavefaciens* MD-1 into *Escherichia coli* using plasmid pBR322 and designated as pEndA Restriction mapping using *Bam*HI, *Eco*RI, *Pst*I and *Hind*III revealed that the cloned fragment contains the restriction sites for *Bam*HI and *Eco*RI Double digestion of *Bam*HI and *Eco*RI produced 1 3 and 3 8 kbp fragments from the cloned DNA insert Only the 3 8 kbp fragment expressed endoglucanase activity when subcloned in *E. coli* using plasmids pUC18 and pUC19 and the cloned plasmids were designated as pEndB and pEndC respectively The 3 8 kbp endoglucanase gene was expressed in both orientations in pUC18 and pUC19 Southern hybridisation with the 3 8 kbp fragment as a probe against *BamHI* digested genomic DNA of *R. flavefaciens* MD-1 revealed that the cloned endoglucanase gene originated from *R. flavefaciens* MD-1 and not from *E. coli* The endoglucanase specific activities units of *E. coli* containing pEndA, pEndB and pEndC were similar and were found mainly in the periplasmic space of *E. coli* with less than 10% of the activities in the culture supernatant Shuttle vector, pYK4 which was constructed for rumen bacteria, *B. fibrisolvens,* has been successfully transformed into *Butyrivibrio fibrisolvens* OB156 and *B. fibrisolvens* OB156C with transformation efficiency of 10^2 and 10^3 per μg plasmid respectively Plasmid pYK4 was able to replicate in the host

cells and express erythromycin resistance, indicating that the ability of *B. fibrisolvens* to accept foreign gene.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia bagi memenuhi syarat untuk mendapat ijazah Sarjana Sains

KAJIAN KE ATAS BEBERAPA BAKTERIA ANAEROBIK RUMEN DENGAN MERUJUK GEN ENDOGLUKANASE

OLEH

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MAY 1996

PENGERUSI: **PROFESOR HO YIN WAN , PhD.**

FAKULTI: **KEDOKTORAN VETERINAR DAN SAINS PETERNAKAN**

Tujuh bakteria rumen, *Fibrobacter succinogenes* D1, D3, D5 dan D6, dan *Ruminococcus flavefaciens* MD-1, MD-8 dan MD-9 dapat menghadamkan gentian dalam cebisan kertas turas Whatman No.1 dalam media Scott dan Dehority dengan aktif. *F. succinogenes* D3 dan *R. flavefaciens* MD-1 adalah paling aktif di antara tujuh strain dan digunakan untuk analisis enzim endoglukanase. *R. flavefaciens* MD-1 menghasilkan aktiviti endoglukanase yang lebih tinggi berbanding dengan *F. succinogenes* D3 pada semua peringkat pengeraman. Elektroforesis gel poliakrilamida (PAGE) menunjukkan bahawa kedua-dua *F. succinogenes* D3 dan *R. flavefaciens* MD-1 masing-masing mempunyai 3 isoenzim endoglukanase dengan jalur pada

kedudukan Rf 0.40, 0.49 dan 0.61; dan 0.66, 0.74 dan 0.84. Satu fragmen gen berukuran 5.1 kbp mengekspresi endoglukanase dari *R. flavefaciens* MD-1 telah diklon ke *Escherichia coli* dengan menggunakan plasmid pBR322 dan dikenali sebagai pEndA. Pemetaan gen menggunakan enzim-enzim pembatas *Bam*HI, *Eco*RI, *Pst*I dan *Hind*III menunjukkan bahawa fragmen yang diklonkan mempunyai tapak pemotong bagi enzim *Bam*HI dan *Eco*RI. Pemotongan berkembar menggunakan *Bam*HI dan *Eco*RI menghasilkan fragmen bersaiz 1.3 kbp dan 3.8 kbp dari selitan DNA. Hanya fragmen 3.8 kbp sahaja yang mengekspresikan aktiviti endoglukanase setelah disubklonkan ke *E. coli* dengan menggunakan plasmid-plasmid pUC18 dan pUC19 dan masing-masing dikenali sebagai pEndB dan pEndC. Gen endoglukanase bersaiz 3.8 kb diekspresikan melalui dua orientasi di dalam plasmid-plasmid pUC18 dan pUC19. Aktiviti endoglukanase yang dihasilkan oleh *E. coli* membawa pEndA, pEndB dan pEndC adalah sama, di mana kebanyakan enzim didapati di ruang periplasmik *E. coli* dan kurang daripada 10% aktiviti endoglukanase di dalam supernatant kultur. Plasmid pYK4 yang telah dibina untuk membawa gen ke dalam bakteria rumen, *B. fibrisolvens,* telah berjaya ditransformasi ke dalam *Butyrivibrio fibrisolvens* OB156 dan *B. fibrisolvens* OB156C masing-masing pada frekuensi 10^2 dan 10^3. Plasmid pYK4 dapat berreplikasi di dalam sel hos serta mengekspresi kelalian terhadap antibiotik erythromycin, menunjukkan kesediaan *B. fibrisolvens* menerima gen asing.

CHAPTER I

INTRODUCTION

Ruminants are grazing mammals that consume mainly fibrous plant materials. The ability of ruminants to utilise fibrous materials for energy depends on the symbiotic interaction between the microorganisms in the rumen and the host animal. The rumen is the largest compartment of the fore-stomach that provides a suitable environment for growth of a large population of fiber degrading microorganisms. The rumen microbes, which consist mainly of bacteria, fungi and protozoa, are capable of producing fibrolytic enzymes to degrade fibrous materials and assimilate the hydrolytic products for their own growth. In return, the host animal is supplied with nutrients produced by the rumen microorganisms as end-products and by-products. Some of the microbes that are washed into the abomasum and lower tract are digested and serve as protein source for the host. The interactions, although beneficial most of the time, are not so favorable in some situations. The degradation of highly fibrous materials is often slow and inefficient. This is very significant in some countries where the main feeds for ruminants are fibrous agricultural by-products. Cellulose is one of the major components of the fibrous feed materials. This polymer is very resistant to degradation because of the strong lattice structure of the cellulose fibres that are held tightly together by hydrogen bonding forming the insoluble crystalline cellulose. The slow degradation of fibrous materials reduces the fermentation rate and the supply of

nutrients to the host animal. This inefficiency in feed conversion has led to the production of low quality meat and milk.

In the past few decades, much research has been carried out to improve the degradation of feeds in the rumen. Some of the approaches taken were by treating the feed materials chemically and physically or by supplementing various non-fibrous feed. This has improved rumen fermentation to some extent, but has not attained the desired level. Another possible approach is to enhance the fibrolytic activities of the rumen microbes by using genetic engineering. All truly fibrolytic bacteria such as *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens* and *Clostridium thermocellum* possess a group of cellulase and xylanase genes (Shellhorn and Forsberg, 1984; Beguin, 1990). These genes code for multiple endoglucanases and xylanases that solubilised fibrous materials more completely than any other rumen microorganisms *in vitro*. This is due to the fact that endoglucanases and xylanases secreted by the fibrolytic microorganisms consist of several domains. Some enzymes have multifunctional domains, while others have separate catalytic and binding domains and some have multiple catalytic domains. These fibrolytic bacteria serve as a potential source of highly active cellulase genes for cloning.

The rumen bacteria have been reported to secrete multiple endoglucanase enzymes originated from different genes. A good example is *R. albus* where 10 endoglucanase genes with different sequences coding for different endoglucanases were cloned (Kawai et al., 1987; Howard and White, 1988; Ohmiya et al., 1988; Romaniec et al., 1989; Ware et al., 1989).

There are many highly fibrolytic rumen bacteria isolated from ruminants in the tropics However, there is limited information pertaining to their molecular characteristics, especially the endoglucanase and xylanase genes Therefore, this study was conducted to investigate some molecular characteristics, particularly the endoglucanase gene, of the rumen bacteria, *F. succinogenes* and *R. flavefaciens,* isolated from ruminants in the tropics

The specific objectives of this study were

(1) to study the morphology of *F succinogenes* and *R flavefaciens* and to assay their endoglucanase activity,

(2) to analyse the endoglucanase of *F succinogenes* and *R. flavefaciens* using polyacrylamide gel electrophoresis (PAGE) and select one strain with the highest fibrolytic activity for cloning the endoglucanase gene,

(3) to optimise various methods for cloning endoglucanase gene from the selected rumen bacterial strain,

(4) to clone the genomic DNA which was fragmented with restriction enzymes and to construct a genomic library,

(5) to screen for endoglucanase positive clone(s) from the genomic library,

(6) to study the expression of the cloned gene and to do restriction mapping, and

(7) to transform the rumen bacteria, *Butyrivibrio fibrisolvens,* using a newly developed pYK4 plasmid, which acted as a shuttle vector for rumen bacteria

CHAPTER II

LITERATURE REVIEW

The Rumen Microorganisms

The rumen is inhabited by various microorganisms consisting of obligate and facultative anaerobes. The major groups of anaerobic microorganisms that play a role in fiber degradation are the bacteria, fungi and protozoa. The rumen is normally dominated by the bacteria. However, the populations of microorganism often fluctuate. For instance, the number and types of bacteria found could be very different before and after feed intake. The microbial species can also change according to the host diet. The rumen fungi are found abundantly in the rumen of animals fed high-fibre diets but are almost absent in animals fed low fibre or starchy feed (Bauchop, 1979; Kostyukovsky et al., 1991). The ciliate protozoal population may be as high as 40% of the total microbial nitrogen in the rumen when the conditions are ideal (Hungate, 1966).

Rumen Bacteria

Rumen bacteria are the predominant and most important microorganisms in the rumen because of their ability to survive unfavorable conditions and to produce a complete range of enzymes that degrade structural components of plant cell wall. The rumen bacterial population may reached 10^{10} -10^{11} cells /ml through direct counting of rumen fluid (Trinci et al., 1994). Basically, the rumen bacteria are categorised into 4

groups, the bacteria that are free in the rumen fluid, bacteria that attach to the feed particles, bacteria that attach to the ruminal wall and bacteria that attach to protozoa (Preston and Leng, 1987) More than 29 genera of rumen bacteria have been identified, including the three major fiber degrader, *F. succinogenes*, *R. albus* and *R. flavefaciens* (Steward and Bryant, 1988). The fibrolytic rumen bacteria can be grouped according to their ability to degrade various parts of the fiber (Table 1).

Rumen Fungi

The anaerobic fungi were discovered in the rumen only about two decades ago (Orpin, 1975; Bauchop, 1979). The rumen fungi are divided into two general groups, the monocentric and the polycentric. There are three genera in the monocentric group, viz., *Neocallimastix*, *Piromyces* and *Caecomyces*, and two in the polycentric, namely, *Orpinomyces* and *Anaeromyces*. All the rumen fungi discovered reproduce asexually. The life cycle of the anaerobic fungi essentially consists of a motile zoospore stage, moving freely in the ruminal fluid, and a non-motile stage consisting of sporangia and rhizoidal or hyphal system attached to the plant particles (Heath et al., 1983; Gold et al., 1988). The monocentric fungi produce a single sporangium and an anucleate rhizoidal system and the polycentric fungi produce an extensive network of rhizomycelium with many sporangiophores on which sporangia develop.

Table 1

Fibrolytic Rumen Bacteria

Fibrous material	**Species**
Cellulose	*Fibrobacter succinogenes*
	Ruminococcus flavefaciens
	Ruminococcus albus
	Butyrivibrio fibrisolvens (certain strains)
	Clostridium lochheadii
	Cillobacterium cellulosolvens
	Cellulomonas fimi
	Eubacterium sp.
Hemicellulose	*Ruminococcus flavefaciens*
	Ruminococcus albus
	Butyrivibrio fibrisolvens
	Prevotella ruminicola
Pectic substance	All the cellulolytic and hemicellulolytic species plus:
	Lachnospira multiparas
	Streptococcus bovis
	Succinovibrio dextrinosolvens

Source: Cheng et al., (1984).

Rumen fungi have been suggested to play an important role in the degradation and subsequent fermentation of fibrous materials in the rumen (Mountfort and Asher, 1985; Pearce and Bauchop, 1985; Wood et al., 1986). The rumen fungi produce a

range of cellulolytic enzymes, namely, endoglucanase, exoglucanase and β-glucosidase, that act synergistically to break down crystalline cellulose (Mountfort and Asher, 1985; Pearce and Bauchop, 1985; Wood et al. 1986; Wood, 1991). Anaerobic fungi are among the world's highly cellulolytic fungi. *Neocallimastix frontalis* co-cultured with a rumen methanogen produce active cellulases that solubilise 98% of the highly ordered cotton, which is more active than the cellulase produced by *Trichoderma reesei* (C-30), one of the highest cellulolytic fungi known (Wood et al., 1986).

The metabolism of anaerobic fungi produces end-products such as formate, acetate, lactate and ethanol (Bauchop and Mountfort, 1981). The contribution of the anaerobic fungi, however, is still unclear as there is difficulty in measuring accurately their biomass in the rumen, although *in vitro* studies have shown that some of the fungi are relatively active fibrolytic microorganisms (Bauchop and Mountfort, 1981; Mountfort and Asher, 1985; Pearce and Bauchop, 1985). Orpin (1981), has estimated the fungal biomass in the rumen to be around 8%.

Rumen Protozoa

The rumen protozoa have been found to contribute about 25-30% of the total fibre degradation (William and Coleman, 1992). The population of rumen protozoa is between 10^5 -10^6/ml (Ogimoto and Imai, 1981). There are two classes of protozoa; ciliates and flagellates. Ciliates are classified into two main groups: holotrich and