Recent Advances in Gut Microbiology and Their Possible Contribution to Animal Health and Production - A Review -

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ABSTRACT : Although gut microbial functions have been analyzed through cultivation of isolated microbes, molecular analysis without cultivation is becoming a popular approach in recent years. Gene cloning studies have partially revealed the mechanisms involved in fiber digestion of individual microbe. The molecular approach finally made it possible to analyze full genomes of the representative rumen cellulolytic bacteria *Fibrobacter* and *Ruminococcus*. The coming database may contain useful information such as regulation of gene expression relating to fiber digestion. Meanwhile, unculturable bacteria are still poorly characterized, even though they are main constituents of gut microbial ecosystem. The molecular analysis is essential to initiating the studies on these unculturable bacteria. The studies dealing with rumen and large intestine are revealing considerable complexity of the microbial ecosystems with many undescribed bacteria. These bacteria are being highlighted as possibly functional members contributing to feed digestion. Manipulation of gut bacteria and gut ecology for improving animal production is still at challenging stage. Bacteria newly introduced in the rumen, whether they are genetically modified or not, suffer from poor survival. In one of these attempts, *Butyrivibrio fibrisolvens* expressing a foreign dehalogenase was successfully established in sheep rumen to prevent fluoroacetate poisoning. This expands choice of forages in tropics, since many tropic plants are known to contain the toxic fluoroacetate. This example may promise the possible application of molecular breeding of gut bacteria to the host animals with significance in their health and nutrition. When inoculation strategies for such foreign bacteria are considered, it is obvious that we should have more detailed information of the gut microbial ecology. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 6 : 877-884)***

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INTRODUCTION

The domestication of herbivore species dates back as far as 10,000 years ago, and herbivores still provide products that are useful in our daily lives. Over the past 50 years, animal production has been markedly enhanced as a result of genetic selection, as well as improvements in feeding and management. However, animals likely have potential for even greater productivity, which may be partly achieved by manipulating gut microbes, including bacteria, fungi and protozoa.

Herbivorous animals are either foregut (rumen) or hindgut (large intestine) fermenters and depend heavily on microbial fermentation within their gut to acquire energy from plants (Forsberg et al., 1997). Possible ways to improve animal productivity include optimizing the functioning of existing microbial ecosystems, or manipulating these ecosystems through modern technology. Therefore, it is important to evaluate the functions of microbes, as well as the significance of these functions within natural ecosystems, in order to determine how to best optimize productivity. Molecular biology has made significant contributions to the study of microbes and their functions, allowing specific microbes to be analyzed in a quick, sensitive and accurate manner, regardless of whether or not they can be cultured (Gregg et al., 1996; Kobayashi and Onodera, 1999; McSweeney et al., 1999; Forano and Flint, 2000).

As our understanding of individual gut microbes and the microcosms in which they exist has progressed, genes encoding the enzymes produced by gut microbes have been cloned, characterized, and used to enhance bacterial function, as well as to track specific bacteria. The continued application of molecular biological techniques might lead to further improvements in the gut functioning of herbivores, particularly with regard to fiber digestion, microbial protein synthesis and detoxification, once it is learned how to best manipulate gut microbial ecosystems.

This mini-review describes the newly revealed functions and ecology of gut microbes as a result of application of molecular biology and how this information might be applied to the improvement of animal health and productivity.

GUT MICROBIAL FUNCTION AND ECOLOGY

Culturable microbes

The various functions of gut microbes have been

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Table 1. Characteristics of culturable cellulolytic bacteria in animal gut

Cellulolytic bacterial species	Polysaccharide utilization				Cellulose attachment (%)**		Distribution	
	Cellulose	Xylan	Pectin	Starch	Crystaline	Microcrystaline	Cattle/sheep*	Horse***
Fibrobacter succinogenes	+	-	v	v	50	120	d	d
Ruminococcus albus	+	+	NA	-	80	101	d	d
Ruminococcus flavefaciens	+	+	NA	-	7	88	d	d
Butyrivibrio fibrisolvens	v	+	+	v	3-78	26-108	d	d
Eubacterium cellulosolvens	+	+	NA	-	77	107	d	NA
Prevotella ruminicola	-	v	v	v	NA	NA	d	d
Eubacterium ruminantium	-	v	v	-	NA	NA	d	NA

+: ferment. -: not ferment. v: variable. NA: data not available. d: detected.
* Forsberg et al. (1997) ** Rasmussen et al. (1989) *** Koike et al. (2001); Yamano et al. (unpublished).

evaluated through cultivation of isolated microbes. Cultivation generates sufficient quantities of selected microbes to determine their chemical composition (i.e., GC content and long-chain fatty acid composition), and for a series of biochemical tests (i.e., sugar fermentation profiling, etc.) to be performed, both of which are traditional means of microbial identification (Minato et al., 1990). Also, cultivation of a particular microbe allows further characterization of the individual microbe in the gut as shown in Table 1, which illustrates the physiological and ecological functions of several representative cellulolytic bacteria.

A number of genes important to fiber degradation, ammonia assimilation, and lactate utilization, have been characterized using culturable microbes, including bacteria and fungi. Ha et al. (2001) and Jun et al. (2003) have illustrated the significance of debranching enzymes in fiber digestion by characterizing several enzymes from *Fibrobacter succinogenes* and rumen fungi. In addition, regulation of the gene expression of fibrolytic enzymes has been partially determined in *Prevotella* (Miyazaki et al., 2003). The presence of cellulosome, a cellulase complex, in *Ruminococcus albus* and *Ruminococcus flavefaciens* has also been suggested by molecular characterization (Karita et al., 1997). The ability to purify target proteins and clone and express them in *E.coli* has led to these discoveries.

Likewise, this approach has made it possible to analyze the genomes of two representative cellulolytic bacterial species from the rumen of *F. succinogenes* and *R. albus* (White and Morrison, 2001). This project organized by North American Consortium is closer to an end. It is hoped that this database will contain useful information regarding the regulation of genes responsible for fiber digestion upon its completion.

Even from protozoa that cannot be cultured *in vitro* but rather must be harvested from the guts of mono-faunated ruminant animals, novel genes involved in fiber digestion are being characterized by mRNA isolation followed by cDNA synthesis. Although the actual contribution of these genes to ruminal fiber digestion has yet to be established, some species of rumen protozoa, such as *Epidinium* and *Polyplastron*, have genes encoding cellulase and hemicellulase, indicating that they have cellulolytic activity (Devillard et al., 1999; Takenaka et al., 1999; Santra and Karim, 2003). Presently, protozoal functions are being extensively explored through the dEST project (Newbold et al., personal communication), which may help to clarify the nutritional significance of individual protozoal species in ruminants. Identification of useful genes from protozoa and/or other gut organisms might lead to their use as food additives or probiotics (Singh et al., 2001).

Molecular approaches have also provided new insight into gut microbial ecology. Representative cellulolytic bacteria, namely *F. succinogenes*, *R. albus* and *R. flavefaciens*, can be tracked without cultivation using DNA probes (Weimer et al., 1999), competitive PCR (cPCR) (Koike and Kobayashi, 2001) and real-time PCR (Tajima et al., 2001). The ability of a particular microbe to attach to cellulose fibers can be determined using pure cultures of each strain, as shown by the results depicted in Table 1. However, this can also be determined using mixed cultures by collecting fibers to which bacteria have attached and analyzing the bacterial DNA (Koike et al., 2003a,b). This more closely reflects actual bacterial attachment during digestion in the gut, in which competition for attachment among different microbes likely occurs.

Unculturable microbes

Molecular techniques are increasingly utilized in the investigation of gut microbial ecology. Several

Table 2. Methodologies to investigate microbial community of animal gut

Methods for enumeration	Sample preservation	Laboriousness	Detailed classification	Unculturable microbes	Quantitivity	Other characteristics	Literatures
Direct counting	Yes	Low	No	Yes	(High)*	allows no useful grouping	Minato et al.(1990)
Viable counting	No	High	No	No	Low	covers 10-50% of total population	*ibid.*
DNA probing	Yes	Low	Yes	Yes	High	needs target at 10^{6-7}/g	Weimer et al.(1999)
Competitive PCR	Yes	Low-medium	Yes	Yes	Very high	needs target at 10^{3-4}/g	Reilly and Attwood (1998)
Real time PCR	Yes	Low	Yes	Yes	Very high	needs expensive machine	Tajima et al. (2001)
Sequencing	Yes	Medium-high	Yes	Yes	Low	produces PCR bias	Whitford et al. (1998)

* Highly quantitative for total or morphologically different population.

Table 3. Gut microbial community revealed by sequencing analysis of 16S rRNA gene library

Source	Proportion of bacteria grouped in different similarity level (%)			Proportion of LGCGP:CFB:others *	Literatures
	>97%	97-90%	90%>		
Cow (rumen digesta)					Tajima et al.
Liquid fraction	2	40	55	48:38:14	(1999)
Solid fraction	10	36	55	69:29:02	
Sheep (hay suspended in the rumen)					Koike et al.
Alfalfa with attached bacteria	33	29	38	48:29:23	(2003b)
Orchardgrass with attached bacteria	11	41	48	42:54:04	
Horse (large intestinal whole digesta)					Daly et al.
from unknown breed	11	77	12	72:20:08	(2001)
Horse (fresh feces)					Yamano et al.
from Thoroughbred	0	29	71	60:40:00	(unpublished)
from Native pony	4	29	67	37:52:11	

methodologies, such as 16S rRNA gene sequencing, probe hybridization, quantitative PCR, T-RFLP and DGGE, have been adopted for this purpose. These techniques have removed the necessity for labor-intensive works such as isolation and cultivation of microbes followed by a number of biochemical tests and have also enabled long term storage of digesta samples (Table 2). For this reason, even wild animals, captured at a distance from laboratories, are becoming targets of new investigations. Studies regarding the microbial ecosystems of the bovine rumen and equine large intestine have revealed that both ecosystems have considerable complexity and contain a number of yet uncharacterized bacteria (Table 3). These are known as VNC (viable but not culturable) bacteria and may play an important role in fiber digestion.

Lin et al. (1995) have revealed that the equine intestine has an abundance of *Fibrobacter*, many strains of which have never been described, isolated or cultivated. Recently, Daly et al. (2001) have reported extensive bacterial diversity in the equine large intestine, with unrecognized sequences being found in a majority (89%) of 16S rRNA genes cloned from equine large intestinal digesta.

Tajima et al. (2000) have reported that rumen flora, analyzed by comparative sequencing analysis of the 16S rRNA gene library, shifts to a simpler one with decreased number of undescribed sequences when a high-concentrate diet is fed. They also found that uncultured lactic acid-utilizing bacteria could be isolated and cultured from the rumen fluid of cattle on high concentrate diets. Phylogenetically speaking, these bacteria were between *Selenomonas* and *Mitsuokella*, possibly marking a novel group of bacteria (Tajima et al., unpublished results). Attempts are being made to further characterize these bacteria so that they might be used as a source of lactate-utilizing enzymes or lactate-utilizing enzyme genes.

Koike et al. (2003b) found that 16S rRNA genes varied when they were isolated from rumen bacteria attached to hay stem, depending on the type of hay used. Since fiber-attaching bacteria are thought to be involved in fiber degradation or metabolite utilization of degraded fiber, the bacteria described by Koike et al. must be directly or indirectly involved in fiber digestion. Thus, even among fiber-attaching flora, considerable complexity is seen. This suggests that presently recognized fibrolytic species, such as *F. succinogenes* and the two ruminococci species known to have fibrolytic activity, may represent only a small proportion of the total fibrolytic population. It will be important to quantify and culture these undefined bacteria to illustrate this and to fully understand the ecology of fiber

Table 4. Fate of newly introduced bacteria into the rumen (1998-2002)

Introduced bacteria	Fate in the rumen	Tracked by	Literatures
Recombinant			
B. fibrisolvens	established at 10^{6-7}/g *in vivo*	PCR	Gregg et al. (1998)
expressing	as combined population of recombinants		
dehalogenase	(protective effect on fluoroacetate poisoning of sheep)		
(mixture of 4 different recombinants used)			
Recombinant			
B. fibrisolvens	decreased to none or 10/g *in vitro*	PCR	Krause et al. (2001)
expressing	decreased from 10^6 to 10^3/g during 22d *in vivo*		
foreign xylanase	(no positive effect on forage digestibility)		
Recombinant			
B. fibrisolvens	maintained at 10^{4-5}/g for 2d *in vitro*	Cultivation and	Kobayashi et al.
expressing	disappeared in 7d *in vivo*	Competitive	(2001, 2002)
foreign xylanase	(no functional assessment)	PCR	Kobayashi et al. (2000, 2001)

digestion. Meanwhile, cPCR assays (Koike et al., 2000; Koike and Kobayashi, 2001; Koike et al., 2003a) have revealed that *F.succinogenes* form a much greater proportion of the sheep rumen and horse hindgut population than the two fibrolytic ruminococcal species (10^{8-9} vs. 10^{6-7}/g of digesta). This suggests that, at the very least, *F.succinogenes* plays an important role in fiber digestion in these animals. The same technique was also successfully applied to the detection of *F. succinogenes* and *S. bovis* population shift after diet change, and *F. succinogenes* attachment to the fiber under different pH (Kim, 2004).

LINKAGE TO ANIMAL HEALTH AND PRODUCTION

Tolerance to toxic agents

Efforts to manipulate gut bacteria and the ecology of the gut to improve animal productivity are still met by many challenges. Bacteria often show poor survival when newly introduced to the rumen, regardless of whether or not they have been genetically modified. However, Allison et al. (1990) have found a way to prevent mimosine poisoning through the successful transfer of functional bacteria. Transfer of rumen fluid from mimosine-tolerant Hawaiian goats to intolerant Australian sheep has enabled the sheep to develop tolerance to mimosine-containing plants, which are widely distributed throughout Australia. *Synergistes jonesii*, which degrades 3,4-DHP, a metabolite of mimosine, was later found to be responsible for this outcome (Allison et al., 1990).

Similarly, isolation of monofluoroacetate-degrading microorganisms has been attempted from fluoroacetate-tolerant wild herbivores, in order to inoculate intolerant sheep. However, all efforts to isolate bacteria that might be used to induce tolerance to this toxic compound, which exists in tropical and sub-tropical plants, have been unsuccessful (Kopecny, personal communication). Gregg et al. (1994) chose GMO as an alternative to the use of indigenous microbes and have produced recombinant *Butyrivibrio fibrisolvens* expressing dehalogenase, from the soil bacterium *Moraxella* species. These recombinant bacteria were successfully established in the sheep rumen, preventing fluoroacetate poisoning in selected sheep (Gregg et al., 1998). Advancements such as this promote tolerance to a greater range of plants, many of which contain these toxic compounds.

Tannin often accumulates in the leaves and seeds of various plants, inhibiting gut digestion by producing indigestible complexes with plant proteins. Although tannin production is considered a natural form of defense against animal browsing, some herbivores have developed the ability to degrade tannin-protein complexes originating from gut bacteria. This has been observed in koalas (Osawa, 1990) and domesticated ruminants fed tannin-rich shrubbery (McSweeney et al., 2001). Biochemical characterization and gene cloning of tanninase from animals with such indigenous functional bacteria might allow the enzyme to be used to enhance animal production.

Enhancement of fiber digestion

Enhancement of gut fibrolysis has been a central topic in the field of herbivorous animal nutrition. Although improved animal feeding and management have increased fiber digestion in ruminants to some extent, more drastic changes can be expected by gut microbial manipulation, including the genetic modification of gut bacteria (Kobayashi, 2003). Up until now, genetic manipulation has been largely focused on the development of highly fibrolytic bacteria, for which main experimental tools concerning gene transfer and expression (i.e., host-vector systems) are available. The basic strategy has been to enhance the cellulolytic and hemicellulolytic activity of moderate to weakly fibrolytic rumen bacterial species. Two such species are *B. fibrisolvens* and *P. ruminicola*, both of which are major constituents of the bacterial flora and less

sensitive to conditions of low pH than the highly fibrolytic *Fibrobacter* and ruminococci species, which lose their activity at low pH. These host profiles might be advantageous for the establishment at higher density and for survival in the rumen of animals on high concentrate diets.

So far several research institutes have produced highly fibrolytic rumen bacteria (Daniel et al., 1995; Whitehead et al., 1995; Coppa et al., 1997; Xue et al., 1997; Kobayashi et al., 1998; Kam, 2000; Krause et al., 2001; Kobayashi et al., 2003), some of which are capable of digesting natural, as well as purified and synthetic cellulose. These bacteria efficiently secrete potent fibrolytic enzymes which benefit extracellular fiber degradation. Xue et al. (1997) and Kobayashi et al. (2003) have developed secretion cassettes for xylanase and cellulase using signal peptide-coding DNA sequences from *B. fibrisolvens* amylase and xylanase, respectively. Thus, genetically altered rumen bacteria can secrete their engineered products in culture, indicating that individual recombinants may have the potential to digest plant material *in vivo* (Krause et al., 2001). However, to date there is no proof that such recombinants can survive and enhance fiber digestion after being released into the animal gut.

Survival of newly introduced bacteria

Recent reports concerning the survival of recombinant rumen bacteria are summarized in Table 4. These new bacteria usually show poor survival or fail to become established in the animal gut. Previously, we examined factors that might inhibit the survival of genetically altered *B.fibrisolvens* in the rumen (Kobayashi et al., 2001; Kobayashi and Yamamoto, 2002). The success of inoculating sheep with our recombinant *B. fibrisolvens* was dissapointing, with ruminal density falling to undetectable levels within 7 days. Judging from the fact that even cell-free rumen fluid quickly inhibits the survival of recombinant bacteria *in vitro*, it is possible that anti-bacterial factors, such as bacteriocin, exist in the supernatant of rumen fluid and act as major inhibitors of survival. Importantly, inoculation of large amounts of recombinant bacteria resulted in accelerated elimination of recombinant bacteria from the rumen. A rapid decrease in the number of inoculated *P. ruminicola* (not recombinant) has also been reported in the rumen of sheep, when the organism was tracked with a DNA probe specific to the strain used (Attwood et al., 1988). Similarly, Varel et al. (1995) have reported unsuccessful inoculation of 6 liters of *Clostridium longisporum* culture into the rumen.

These negative results, however, have become largely ignored in light of the recent breakthrough of Gregg et al. (1998) who succeeded in maintaining recombinant *B. fibrisolvens* in the sheep rumen expressing foreign dehalogenase, which acts to prevent fluoroacetate poisoning. They inoculated several different recombinant strains of *B.fibrisolvens*, some of which survived by out-competing indigenous rumen microbes. As a consequence, sheep with recombinant levels of 10^{6-7}/g were able to tolerate fluoroacetate-supplemented feed. Field trials are now required in order to confirm the feasibility of this GMO project. Thus, successful inoculation of mixed candidate strains may provide possibility of manipulating gut bacteria in a way to improve the health and nutrition of host animals.

Strategy for successful survival

More information is required on the microbial ecosystem in the gut in order to develop successful strategies for novel bacteria inoculation (i.e., what comprises the ecosystem and how the population shifts as conditions vary). Extensive use of new molecular methodologies might clarify these issues. In particular, bacteria-bacteria, bacteria-protozoa, and bacteria-fungi inter-relationships should be highlighted in any analysis of the gut microbial ecosystem, since some microbes are thought to be dependent on others for growth. This may be one of the reasons why some microbes cannot be cultivated into pure cultures. Koike et al. (2003b) have found that some of cloned 16S rRNA genes from hay stem-attaching bacteria belong to a group of bacteria that share phylogenetic similarities with *Treponema* in that they are unable to digest fiber but can grow on fiber with the aid of *Fibrobacter*. Although strains belonging to this group have not yet been cultivated, molecular analysis without cultivation has demonstrated the symbiotic nature of this group of bacteria, and similar phenomenon may occur widely throughout the gut. It is undoubtedly important to identify examples of microbial symbiosis, and also the effects of competition among small niches, before manipulation of the animal gut ecosystem is attempted.

With regard to the inoculation of functional microbes, it seems obvious that microbes must be established within the gut at a satisfactory level (10^{8-9}/g) to have an impact on overall gut fermentation. Recently, a promising strategy for selection of genetically altered bacteria within the rumen has been proposed. As part of this strategy, bacteriocin-producing host strains, which generally target phylogenetically close relatives, are selected for genetic alteration (Teather and Forster, 1998). Butyrivibriocin, a bacteriocin produced by *B. fibrisolvens*, has a relatively narrow spectrum of antibacterial activity, acting primarily against other *B. fibrisolvens* strains (Teather et al., 1997) occupying the same micro-niche. If successfully established *in vivo*, the bacteriocin-producing strain, whether it is native or genetically altered, would have a competitive advantage against indigenous strains that live in close proximity.

There is no doubt that numerous recombinant gut

bacteria will be produced in the future, as more information regarding useful genes and gene transfer systems is accumulated. In order to evaluate their fitness in the gut, a quick screening system is surely needed to assess their competitive ability in the gut. This can be measured through substrate attachment, anti-factor tolerance and growth. Recently developed tracking methods, in particular cPCR and real-time PCR, allow these factors to be evaluated in a quick, sensitive and accurate manner, even in mixed cultures. The properties measured by PCR-aided methods facilitate the selection of target microbes, either natural or recombinant, for the promotion of gut function. These molecular approaches also may bring disuse of the antibiotic resistance gene as a selective marker of recombinant microbes. Spread of antibiotic resistance among indigenous microbes has been suspected when a recombinant possessing the marker gene is released into the animal gut.

FUTURE DIRECTIONS

Once successful manipulation of gut function is achieved, its influence on animal health and production becomes of interest. Accumulated data on ionophore antibiotics have demonstrated that gut function can be markedly altered. These propionate-enhancing agents have been widely accepted for use in the beef industry with noted improvements in feed conversion ratios (5-20%) and reduced concentrate intakes (Kobayashi et al., 1990). However, long term use of ionophore antibiotics has led to the development of resistance and thus, decreased potency. In addition, the widespread use of antibiotics among livestock is not well received by consumers these days. Obviously, alternative methods by which to enhance animal productivity should be explored.

One such alternative, the use of naturally occurring microbes or probiotics, has attracted a lot of attention. Some probiotics have been shown to promote weight gain in calves, milk yield in lactating cows, and fiber digestion in the rumen, possibly by creating anaerobic conditions in the gut and stabilizing the microbial population (Wallace, 1992). Lactate-producing bacteria have already been highlighted as health-promoting probiotics in the human and animal gut (Singh et al., 2001), which indirectly support animal production.

As discussed in the present review, gut microbial manipulation has tremendous potential of improving animal health, nutrition and subsequent production. However, it is fairy difficult to quantitatively evaluate how ongoing basic research might contribute to the improvements of animal productivity, because we are still in the early stages of research and face many challenges. Since, theoretically, gut function might be improved by targeting either specific microbes or the ecosystem of the gut, individual microbes and the environment in which they live should be examined in conjunction. Both microbial genetics and ecological perspectives are needed to achieve the goal of improving herbivorous productivity by the way of rumen microbial manipulation.

The ongoing genome project may clarify how fiber is digested by particular species. This may lead to more sophisticated genetic engineering of rumen bacteria. For example, bacteria could be engineered to express a target gene at a specific point of time, using knowledge gained from the genome project regarding regulatory mechanisms. This would allow the engineered bacterium to conserve its energy until required, which would clearly give it a competitive advantage over other indigenous bacteria requiring more nutrients to meet their energy needs. Meanwhile, Teather (personal communication) proposed a metagenomic approach in which various microbes of the rumen would be considered as a single system and genes actually expressed in the rumen could be examined. This has significant potential to identify novel functional genes that are expressed by known species or unculturable microbes. The information gained from such projects would surely enhance our understanding of digestion in the rumen and other animal guts, thereby allowing us to manipulate digestion in ways that improve animal productivity.

DEDICATION

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