BIOTECHNOLOGY RESEARCH AND APPLICATIONS

Edited by

J. GAVORA

D.F. GERSON

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and

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AGRICULTURAL BIOTECHNOLOGY

GENETIC ENGINEERING OF RUMEN BACTERIA FOR IMPROVED PRODUCTIVE EFFICIENCY IN RUMINANTS

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ABSTRACT

The amount of food required to support the high production levels of modern dairy cows is more than the microorganisms which are responsible for feed digestion in the rumen can deal with, at least in the form of fibrous plant material. As a result, high producing animals have to be fed more readily digestible, high energy, high protein diets. Much of the advantage of the ruminant animal, which lies in its ability to use feeds that other animals cannot, is lost. Our long range goal is to use genetic engineering techniques to develop bacterial strains which will improve rumen function so that the needs of high producing ruminants can be largely met by forage or silage diets, with substantial reductions in the addition of protein concentrates or grains.

The genetic manipulation of rumen bacteria relies to a large degree on established recombinant DNA techniques, but new methodologies are required. Potential cloning vectors have been identified, and methods have been developed for the introduction of DNA into several species of rumen bacteria. Our understanding of the biochemistry and regulation of plant fiber degradation in the rumen has been greatly increased by the cloning of genes for enzymes which take part in this process. These same genes provide readily identifiable markers for use in cloning vector development and may provide the means of increasing the rate of this critical process. We are constructing synthetic genes to change the amino acid composition of the rumen bacteria, the animal's major source of dietary protein, so that the microbial protein produced in the rumen will better meet the protein needs of the animal and thus reduce the need for protein supplements in the diet. An artificial rumen has been developed which closely mimics the environment in the bovine rumen. It will provide a controlled, contained environment to test the abilities of genetically modified rumen bacteria.

INTRODUCTION

Historically, ruminant animals have provided farmers with the ability to utilize marginal lands for the production of high quality food for human consumption. Ruminants are able to derive their nourishment from feeds that are not in themselves capable of meeting the nutritional needs of the animal. They do this by supporting in the rumen, which is a greatly enlarged region of the stomach which precedes the animals digestive system, a microbial fermentation system. This system partially degrades complex polysaccharides and provides to the animal not only the degradation products (chiefly volatile fatty acids), but also secondary microbial metabolites and microbial biomass (most importantly microbial protein, which can be synthetized from inorganic nitrogen sources). A ruminant animal is able to survive, if not thrive, on a diet containing only cellulose, a non-protein nitrogen source such as urea, and trace minerals.

The capacity of the rumen to process low quality feeds is limited by factors such as rumen volume, the time required for digestion of these feeds in the rumen, and the ability of the animal to chew the feed. Modern ruminant animals have been intensively selected for high production potential, but the limited capacity of the rumen fermentation means that this potential cannot be realized by feeding the animal low quality feeds. The high production potential of these animals can only be realized by feeding large amounts of readily digestible, high quality feeds such as oilseed meals and grains. Rather than providing an economic advantage in allowing the animal to use low quality feedstuffs, the rumen in this case becomes a liability because a significant proportion of these high quality feeds is degraded in the rumen, with the result that these feedstuffs are not used as efficiently by ruminants as by non-ruminants.

At the present time a major part of the Canadian agricultural economy is based on ruminant animals, chiefly dairy and beef cattle. The dairy and beef cattle industries enjoy total cash receipts of over 6 billion dollars annually, with production costs amounting to about 5 billion dollars. The major cost in production is the cost of feed, amounting to about 3 billion dollars annually, and about half of this amount is devoted to providing feed supplements such as grains and oilseed meals. It is this cost that we expect to reduce by improving the capacity of the rumen fermentation system to meet the nutritional needs of animals with high production potentials.

The capacity of the rumen fermentation system depends on both its physical characteristics and the characteristics of the bacteria which make up a major part of the microbial population of the rumen. The characteristics of these bacteria are subject to selection and modification. In particular, we can apply the powerful techniques of genetic engineering to give new capabilities to these organisms. Just as the production potential of the animals has been increased by animal breeders, we can seek to improve the characteristics of the rumen bacteria so that the capacity of the rumen fermentation to meet the animals nutritional needs with lower quality feeds is increased. The potential saving to the dairy and beef cattle industries by replacing

feed supplements with lower cost forages is about 0.5 billion dollars per year.

GENERAL APPROACHES AND GOALS FOR THE MODIFICATION OF RUMEN BACTERIA

The definition of specific goals for the genetic modification of rumen bacteria is critical for the success of this approach to improving the efficiency of ruminant production. This process has to include the following: 1) the identification, at the biochemical level, of the critical limitations placed on production by the rumen fermentation; 2) the identification or design of the genetic information needed to circumvent these critical limiting steps; 3) the identification of the technical obstacles which must be overcome to apply genetic engineering techniques to the rumen bacteria species involved in these critical steps; and 4) the more general problem of establishing genetically modified bacteria in the complex, highly competitive ecosystem in which the modified bacteria must function.

In general terms the limitations on cost-efficient ruminant production are reasonably well understood. These include: 1) the rate at which lignocellulosic materials can be degraded by the rumen microorganisms; 2) the ability of the fermentation system to maintain conditions conducive to microbial growth (and to animal health) when more readily degraded feeds are used; 3) the composition of the fermentation products and microbial biomass produced in the rumen in relation to the nutritional needs of the animal (particularly the amino acid composition of microbial protein in relation to the amino acid requirements of the animal); 4) the negative effects on the efficiency of feed conversion of specific rumen bacterial metabolic pathways such as methane production; and 5) the inhibitory effects of substances ingested in feed on either the rumen microbial population or the host animal itself. In only a few cases can we describe these limitations in the specific biochemical terms that are required to allow us to plan a solution through the genetic modification of rumen bacteria. In many cases, however, the application of genetic engineering techniques offers the best approach to defining the problems at the required molecular genetic level, as well as to providing a solution.

There are three general routes to the production and selection of rumen bacteria with improved characteristics, which may be combined to meet specific goals: the modification of existing metabolic pathways, chiefly by selection of regulatory mutations in chromosomally encoded genes; the transfer of genes between organisms, which would allow the introduction into the rumen of functions from non-rumen microorganisms; and the design and construction of synthetic genes.

Classical microbial genetic techniques are directed at the modification of chromosomally encoded genes. The modification of existing metabolic pathways by mutation and strain selection has been applied with considerable success in industrial microbial fermentations for the production of secondary metabolites such as amino acids, vitamins, and antibiotics. There are potential applications in this general area for the modification of rumen bacteria, for example in the production of increased levels of specific amino acids whose availability

limits production in milk or wool producing animals and leads in part to the requirement for dietary protein supplements. The nature of the rumen fermentation system, however, imposes severe restraints on this approach. First, products such as amino acids which are excreted by the producing bacterial cells are subject in the rumen environment to immediate degradation by other bacterial species. Second, the process of mutation and selection used to change chromosomally encoded gene functions involves many generations of growth in the laboratory, which inadvertently but inevitably selects for strains capable of more rapid growth under laboratory conditions. This generally entails the loss of functions such as resistance to viruses or bacteriocins, or the ability of the cells to bind tightly and specifically to preferred substrates, or the ability to avoid predation by protozoa, which are critical to the bacteria's establishment and survival in the original ecosystem.

More recently developed genetic engineering techniques provide a way around this obstacle. Genetic information, whether from the host organism, from another organism, or synthetic, can be maintained and manipulated in plasmid or phage vectors in laboratory adapted bacterial strains. The new information can then be transferred in a single step to a recently isolated bacterial strain which has not lost its competitive fitness in the rumen ecosystem.

Genetic engineering techniques could be used to introduce new genetic information into the rumen. A number of specific goals can be identified that can be reached using this approach. For example, antibiotic compounds such as monensin, which are used to improve the efficiency of feed conversion in ruminants, could be produced by bacteria in the rumen rather than being fed in the diet. Gene cloning techniques could be used both to analyze the process of plant fiber degradation in the rumen, by identifying the enzymes and the most limiting steps involved in the process of degradation, and to introduce new genes that overcome these limitations. Synthetic genes could be used to modify the amino acid composition of the microbial protein produced in the rumen so that it more closely matched the amino acid requirements of the animal, effectively decreasing the requirement for supplementary dietary protein.

BARRIERS TO THE GENETIC MANIPULATION OF RUMEN BACTERIA

While there is no reason to doubt that the many genetic systems which have been utilized in well characterized aerobic organisms such as *Escherichia coli* and *Bacillus subtilis* exist and can be used for the genetic modification of rumen bacteria, at the present time these techniques have not been developed to the point of applicability for any of the rumen bacteria species. One reason for this state of affairs is the complexity ot the rumen bacterial population. Another is the technical difficulty of working with the rumen bacteria, almost all of which are obligate anaerobes unable to tolerate even brief exposure to the atmosphere. Whereas with *E. coli* a very large number of scientists worked to develop techniques for a well defined bacterial species, in the case of the rumen bacteria a small number of scientists are working to develop techniques for a very large number of poorly defined species. In fact, the effort to develop techniques for the genetic modification of rumen bacteria has led to a renewed interest in the classification of rumen bacteria using molecular biology techniques. These studies have

shown that in many cases the classification of rumen bacteria has not been adequate in terms of defining the relatedness of these bacteria either among rumen isolates or with better characterized bacteria isolated from other environments. These studies have made it apparent that each rumen bacterial species will probably require the development of its own unique set of techniques for genetic manipulation.

The application of gene cloning techniques to each rumen bacterial species requires three developments: 1) the development of methods for the introduction of a recombinant DNA molecule into the cell (transformation, transduction, electroporation, conjugation); 2) the development of a vector, based on a plasmid, phage, or insertion element capable of functioning in the rumen bacterial species concerned, which will allow stable inheritance of the recombinant DNA molecule in the rumen bacterium; 3) the development of an understanding of the regulation of gene expression in that organism. These three criteria cannot yet be met for any rumen bacteria species, but rapid progress toward these goals is being made. The general problems and prospects for the genetic manipulation of rumen bacteria and related anaerobic bacteria have been reviewed by a number of authors in recent years [1-10].

RECENT PROGRESS

Cloning of genes from rumen bacteria

The cloning of bacterial genes provides a powerful technique for dissecting complex enzyme systems and investigating the regulation of gene expression. The genes may also code for enzymes that can be used to improve rumen function if their rate or site of expression is altered, or contain regulatory regions that can be used to regulate the expression of new genetic material to be introduced into the original organism from which the gene was isolated. We have applied this technique to the isolation of genes involved in fiber degradation in the rumen bacterium Bacteroides succinogenes.

A gene bank was prepared from B. succinogenes and cloned into E. coli. Clones carrying genes of interest were selected on the basis of their ability to degrade carboxymethyl cellulose. Fifteen active clones were isolated, which were later shown to include genes for six different enzymes capable of degrading β-(1→4)-D-glucans [11-13]. The product of one of these genes has been characterized in some detail [14]. Its expression is subject to catabolite repression in E. coli, and the enzyme is transported through the cytoplasmic membrane into the periplasmic space. We have also cloned and characterized a mixed linkage, β-glucanase (a β-(1→3)(1→4)- D-glucanase) from B. succinogenes [15, 16], and have cloned β-glucosidases from Selenomonas ruminantium, Lachnospira multiparus, and B. succinogenes [17].

The isolation and characterization of these genes has provided us with a number of useful tools and much valuable information. The results make it apparent that there are no inherent barriers to the expression of genes from these rumen organisms in a distantly related species, and presumably the converse is also true. We have discovered an unexpected complexity in the enzyme system used for the degradation of cellulose by B. succinogenes. This probably reflects the level of complexity in the

structure of the substrate itself, and means that specific bottlenecks in the degradation of plant fiber in the rumen are likely to exist in the form of specific associated linkages or molecular conformations that require specialized enzymes for degradation. These bottlenecks could be removed by the introduction of appropriate genetic information into the rumen. We now have available the regulatory regions of the cloned genes, which can be used to control the expression of new genetic material introduced into the rumen bacteria which were their source. We also have a gene coding for a readily selected and identified marker enzyme, the mixed-linkage β-glucanase, which can be used in the development of cloning vectors and transformation methods for B. succinogenes. These cloned genes also represent a resource in themselves - a convenient source of enzymatically pure, highly specific polysaccharide degrading enzymes. They require no purification because their new host does not produce any enzymes with related activities. They have potential applications as research tools in revealing the molecular details of plant fiber structure and in analyzing the linkages resistant to degradation in the rumen. They also have potential applications in food analysis and in industries such as brewing and animal feed preparation.

Design and cloning of synthetic genes to reduce the requirement for dietary protein.

The major part of the protein available to the ruminant animal is microbial in origin. Most of this is bacterial protein, produced in the form of bacterial cells by the growth and reproduction of the rumen bacteria during the fermentation of feed in the rumen. These bacteria enter the digestive tract below the rumen together with the other products of the rumen fermentation and are digested by the animal. A large part of the protein fed to the animal is degraded in the rumen and its components are reutilized in the synthesis of bacterial proteins or further degraded to provide substrates for other aspects of microbial metabolism. Unfortunately, the bacterial protein produced in the rumen is not ideally matched to the needs of the animal. In the case of dairy cattle, for example, the supply of the amino acids lysine, methionine, and threonine is limiting. This leads to two types of inefficiencies in protein utilization in these animals. Dietary protein is degraded and converted, with inevitable losses, into microbial protein. The microbial protein itself can only be utilized by the animal for the synthesis of its proteins until the supply of the most limiting amino acid is exhausted. The efficiency of utilization of the microbial protein could be increased, and the need for dietary protein decreased, if the amino acid composition of the microbial protein could be made to more closely match the requirements of the animal. The problem would be difficult to solve by feeding amino acid supplements to the animal, or by creating mutant rumen bacteria that produce increased levels of the limiting amino acids, because free amino acids are degraded very rapidly in the rumen.

We have approached this problem by the synthesis and cloning of a series of synthetic genes. These genes are designed to code for polypeptides containing high levels of the most limiting amino acids. The peptides will remain inside the bacterial cells, protected from degradation, until the cells themselves are digested after passing out of the rumen. The genes prepared to date differ in a number of characteristics, including polarity, net charge, size, and cysteine

content (number of intramolecular disulfide bonds). These genes have been cloned in E. coli.

Our preliminary results indicate that this is a feasible approach to the modification of bacterial amino acid composition. While some of the designed sequences have proved difficult to isolate, indicating deleterious effects on the host cell, others have been isolated and expressed as β-galactosidase fusion proteins using expression vectors based on lac or trp-lac hybrid promoters.

CONCLUSIONS

With the rapidly increasing attention being paid to the development of gene cloning systems for rumen bacteria, progress in this area can be expected to be rapid over the next few years. The recent isolation and characterization of native plasmids from the rumen bacteria Bacteroides ruminicola [7,18], Butyrivibrio fibrisolvens [19-22], S. ruminantium [22,23] Propionibacterium spp. [10], Ruminococcus albus [10,24,25], Ruminococcus flavefaciens [24,25], and Bifidobacterium globosum [26,27] means that the replicons needed to develop shuttle cloning vectors for these species are available. Given these vectors, which can be easily constructed in bacteria such as E. coli, the development of methods for introducing recombinant DNA molecules into rumen bacteria should not be difficult, particularly with the recent availability of commercial electroporation equipment which has been shown to allow the transformation of a wide range of bacterial species without pretreatment.

The more fundamental problems of defining goals for the genetic modification of rumen bacteria at the required molecular level, and of establishing modified rumen bacteria in the highly competitive rumen ecosystem, will be more difficult to solve. The first of these problems can only be solved by carefully focussed research programs. The second problem may in the end prove to be less severe than anticipated because, unlike most natural ecosystems, the rumen ecosystem is recreated in a sterile form with the birth of each new ruminant animal. Colonization of the rumen can be controlled by isolation of the newborn animal, providing the opportunity of excluding competing bacterial strains.

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