APPLICATION OF RECOMBINANT DNA METHODS TO RUMEN BACTERIA

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SUMMARY

The principal aim of this work is to increase the efficiency with which ruminants are able to digest fibrous plant tissues, to allow more effective nutrition of animals grazing on unimproved pastures. The approach taken is to increase the digestive capability of rumen bacteria by genetically engineering them to produce increased levels of cellulase and hemicellulase enzymes, or to produce more efficient combinations of these enzymes.

To this purpose, we have prepared fresh isolates of a wide range of rumen bacteria, to select species that can provide the necessary genes and those suitable as recipients of novel genetic material.

We have prepared libraries of cloned genes, from which cellulolytic and xylanolytic enzyme genes are presently being characterized. Naturally occurring or "native" plasmids have been identified in three genera of rumen bacteria. Genetic information, essential for the replication of these plasmids in their host bacteria, will be inserted into the plasmid pCW1 to produce functional "shuttle" plasmids.

Preliminary experiments have been conducted to show that the process of electroporation can be applied to bacteria to insert plasmid DNA. This method has been selected for transformation experiments on rumen bacteria, using derivatives of pCW1.

While it is evident that the major objectives of this work are of a long-term nature, the initial steps have been taken in several areas of importance. The complexities encountered during these early stages have provided some interesting observations and encouraging early results.

INTRODUCTION

Investigation of the rumen and its micro-organisms has been in progress for a considerable number of years, but the study of the microbial molecular biology of this organ has only very recently been investigated. Our understanding of this topic is sufficiently undeveloped that some contradictory views have been published, on the practicability of recombinant DNA techniques for manipulating the rumen microflora (Teather 1985; Armstrong and Gilbert, 1985). While the practicability of this approach is untested, we believe that the great potential of rumen biotechnology justifies serious efforts to develop the necessary methods.

Our own interest in genetic manipulation of rumen micro-organisms began with the intention of enhancing their ability to digest plant fibre, in order to increase the efficiency with which low grade forages can be used by cattle or sheep. The major gain is expected to be derived from the possibility of

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making fibre breakdown more rapid, thereby allowing a greater voluntary intake of food to provide more of the essential nutrients that are scarce in such diets.

The techniques that must be developed to achieve this aim could find a wide range of applications (see Teather 1985; Smith and Hespell 1982). Examples of these include the modification of bacterial products; suppression of undesirable metabolic pathways; the production of novel substances that may benefit the ruminant; and, possibly, the detoxification of naturally-occurring plant toxins.

In little more than a year, since beginning work in our recombinant DNA laboratory, we have begun to tackle some of the basic problems of this work. Early progress has been encouraging and some of the problems encountered may be of interest to others in the field. The purpose of this overview is to outline the approach taken and to indicate the complexity of the task.

Approach

The approach taken to this project consists largely of proven recombinant DNA techniques, with occasional modifications made necessary by the nature of the bacteria concerned. In describing the entire approach it is inappropriate to give extensive detail of every technique and we would direct interested readers to some of the excellent laboratory manuals that deal with the subject (Maniatis et al. 1982; Glover, 1985). Those methods that have required modification, in order to be applied to rumen anaerobes, will be discussed here more fully.

In outline, the manipulations required to produce enhancement of fibre digestion by rumen bacteria can be divided into seven steps:

1. Isolation of rumen bacteria, both as donors of fibrolytic enzymes and as potential recipients of cloned enzyme genes.

2. Preparation of gene libraries from which genes coding for cellulase and hemicellulase enzymes may be obtained.

3. Identification and characterization of fibrolytic enzyme genes.

4. Identification of additional factors required to endow full fibrolytic capabilities upon the product of these genes and to control their expression.

5. Preparation of plasmid vectors suitable for insertion of DNA into rumen bacteria.

6. Development of processes to insert foreign DNA into rumen bacteria.

7. Development of methods to induce the transfer of plasmid-borne DNA sequences into the genome of rumen bacteria, as stable, heritable characteristics.

Some of these steps require the successful completion of prior steps and cannot be commenced at present. Those sections of the work that have commenced will be discussed in sequence.

Identification of bacteria from the rumen

Despite the availability of cultures of rumen bacteria from sources such as the American Type Culture Collection, it was decided early in the conception of this project to concentrate upon the use of freshly isolated bacterial strains. This decision was based upon the need to be certain that bacterial strains had not lost any of their competitive properties through repeated sub-culturing. In addition it was considered likely that a continuous examination of fresh isolates would yield more actively fibrolytic strains.

Classical taxonomic criteria were used to identify anaerobic bacteria (Holdeman et al. 1977; Ogimoto and Imai 1981) isolated by the methods developed by Hungate (1966). A number of bacterial isolates were further characterized by restriction endonuclease analysis of isolated DNA (Kakoyiannis et al. 1984; Hudman and Gregg, in preparation).

Preparation of gene libraries

High molecular weight DNA was purified from bacterial cultures by conventional means, following lysis of the cells by treatment with lysozyme (1 mg/ml), Proteinase K (25 µg/ml) and Sodium dodecyl Sulphate (0.1%). DNA was partially digested with restriction endonucleases to allow selection of fragments suitable for ligation into plasmid (pUC18) or bacteriophage vectors (λ gtWES.λB). Size selection was by sucrose gradient fractionation or by separation on low melting point agarose gels.

Screening gene libraries for fibrolytic genes

Libraries of DNA clones were screened for the expression of fibrolytic enzyme genes by plating upon agar containing carboxymethyl cellulose or oat-spelts xylan (0.1%) with 0.5% tryptone as a nutritive carbon source. Regions of cellulose digestion were visualized by staining with congo red stain (1 mg/ml) and destaining with 1 M NaCl (Teather and Wood 1982). Simple carbohydrate nutrients such as glucose were specifically omitted from selection plates, to avoid the possibility of enzyme repression. Recombinant clones that produced enzymes capable of digesting carboxymethyl. cellulose were further screened on plates containing 0.2% ball-milled cellulose.

Preparation of cloning vectors

For the construction of cloning vectors suitable for inserting novel DNA into rumen bacteria, the clindamycin resistance gene from the colonic Bacteroides plasmid pDP1 (Guiney et al. 1984) was chosen as a selectable marker. This gene could be excised intact on a 2 kb fragment and was cloned into pUC18 to produce a 4.7 kb plasmid pCW1. This plasmid is the basic unit on which a variety of host-specific plasmids are to be constructed, using replication control sequences derived from naturally occurring or "native" plasmids isolated from rumen bacteria. The final product will therefore contain both replication control sequences and antibiotic resistance selection markers to allow the plasmids to be used in Escherichia coli and in the appropriate rumen bacterium.

Identification and isolation of native plasmids

Total DNA preparations from rumen bacteria were electrophoresed on 1% agarose gels (Tris.borate.EDTA buffer) with approximately 5 µg of DNA per 1 cm track. Small plasmids could be detected as low molecular weight bands,

running ahead of the genomic DNA. Plasmids detected in this way were purified by conventional plasmid isolation techniques (Maniatis et al. 1982).

Insertion of plasmid DNA into rumen bacteria

The process of electroporation involves passing a pulse of electric current across a suspension of bacteria (or other cells) to which a DNA solution has been added. This process has been applied to plant, animal and bacterial cells (Potter et al. 1984; Bio-Rad) and represents the most generally applicable method available for inserting DNA into cells. Preliminary experiments have involved the insertion of a 19 kb plasmid (pDP1 Guiney et al. 1984) into E. coli cells, using pulses from 2 seconds to 10 seconds with an initial potential of 2000 volts. The maximum current passed was determined by the resistance of the suspension medium (0.5 M sorbitol) which gave a current of about 2 mA between electrodes 1 cm apart.

RESULTS AND DISCUSSION

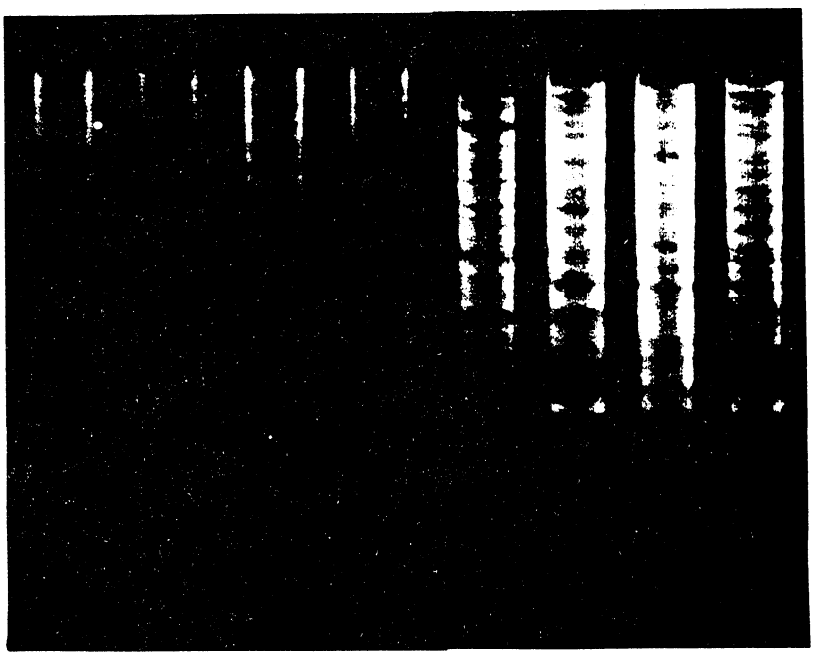
Isolation of bacteria from the rumen

The bacterial strains from which gene libraries are being prepared were recently isolated in our own laboratories and efforts have been concentrated particularly upon cellulolytic and hemicellulolytic species. These include Ruminococcus f lavefaciens, Ruminococcus albus, Bacteroides succinonenes, Bacteroides ruminicola (sub-species ruminicola and brevis) and Butvrivibrio f ibrisolvens, with particular attention being paid to those isolates that demonstrated the highest levels of cellulolytic or hemicellulolytic activity.

Considerable effort is required for the identification of rumen bacteria and in many cases the taxonomy appears to be incomplete (Barnes, 1986; Hobbs, 1986). This problem has been clearly demonstrated by the use of restriction enzyme analysis of purified DNA. Analysis of a series of different isolates of a single species demonstrated that similarity of morphological and biochemical criteria may be accompanied by remarkable differences in restriction endonuclease patterns (Fig. 1). When compared with the restriction patterns obtained from four laboratory strains of E. coli, it is clear that the genetic diversity implied by these results requires a more complete investigation of taxonomic criteria. At present, it is not clear whether the more pronounced differences between restrict ion patterns from B. fibrisolvens are the result of DNA modifications within a single species, or whether this group of bacteria may include genetically distinct species or sub-species.

Construction of gene libraries

A library of cloned DNA fragments, representing the genome of a bacterium, can be satisfactorily constructed using plasmid vectors (Kawai et al. 1987). Between 2000 and 10000 cloned fragments of 4-8 kb would be expected to yield an adequate representation of a bacterial genome, depending upon genome size. This approach was therefore the primary choice for the preparation of gene libraries from rumen bacteria, but turned out to present unexpected difficulties. 'Despite adequate'preparation of component DNA, efficient ligation of foreign inserts with the plasmid vector and acceptable transformation frequencies with the chosen bacterium (E. coli JM109), there was a very poor yield of recombinant plasmid-bearing colonies. Insert ion of the same DNA fragment preparation into both plasmid and bacteriophage vectors yielded poor results with the former and very high cloning efficiency with the



Track 1 2 3 4 5 6 7 8

Fig. 1. Banding patterns produced by restriction enzyme digestion of bacterial DNA and electrophoresis on 1% agarose gels. Tracks 1-4: Pst 1 digestion of DNA from E. coli strains: JM101, JM109, LE392 and MC1061 respectively. Tracks 5-8: EcoR1 digests of DNA from Butyrivibrio fibrisolvens isolates AR9, AR10, AR11, AR12 respectively.

latter. It was concluded that the DNA of some rumen bacteria may be modified in a way that discourages direct transfer of the DNA across the membrane of JM109. Packaging into bacteriophage for active transfer across the membrane appeared to overcome the problem. Interestingly, the use of a different strain of E. coli (C600) has recently allowed us to overcome this problem with plasmid cloning, at least with DNA from some species. At the present time, we have prepared gene libraries from B. ruminicola brevis (a hemicellulolytic species) and from R. f lavefaciens (a cellulolytic species). We are currently also preparing gene libraries fr albus and from a Bacteroides sp. which is potently cellulolytic.

In this project, it will be necessary to obtain fibrolytic enzyme genes from a wide variety of sources, to be certain of detecting those enzymes most suited to production within a foreign host bacterium. Important features include the ability to digest crystalline cellulose and the ability to be regulated by nutrient availability. These properties should, ideally, require the inclusion of few additional genes.

Detection of fibrolytic enzyme genes.

Screening to detect those recombinant clones that produce fibrolytic enzymes is readily performed by plating recombinants on agar that contains carboxymethyl cellulose or xylan and staining with congo red dye. So far, we have detected seven recombinants from R. flavefaciens that digest carboxymethyl cellulose and are screening recombinant clones from B. ruminicola brevis for xylanase activity. Each gene library will, in turn, be screened for all possible fibrolytic enzyme genes, including those concerned with the metabolism of cellulose degradation products such as cellobiose.

An essential part of screening for enzyme activities, is the need to test for regression or inhibition of the activities by simple sugars. Furthermore, the ability to digest carboxymethyl cellulose is, by itself, a poor indicator of the ability to digest natural plant fibres. It is clear that enzymes chosen as candidates for increasing the cellulolytic capabilities of the rumen bacteria must be capable of digesting some of the more resistant (crystalline) components of plant fibres. It is possible that additional genes may be necessary to provide such capabilities. Examples of the genes that may be required include those coding for glycosylases, responsible for post-translational modification of cellulase enzymes, and those responsible for locating the enzyme on the outer cell membrane. Cellular location may be a vital factor in the efficient functioning of such enzymes.

Construction of cloning vectors

Our attempts to construct plasmids that can replicate within rumen bacteria and possess a selectable marker that will allow us to distinguish those bacteria, have centred upon the production of a so-called "shuttle vector". A shuttle vector can be defined as a DNA molecule that is capable of functioning in two or more types of cell. Therefore we have begun the construction of a plasmid that is expected to be useable in both laboratory strains of E. coli and in the chosen recipient strains of rumen bacteria. For these purposes the plasmid must contain DNA sequences that control self replication in both species and also contain selectable marker genes capable of operating in both species (Fig. 2). Shuttle vectors of this type have been constructed recently for use in the colonic species Racteroides fragilis (Smith 1985) and have been used to demonstrate that antibiotic genes that function within E. coli may not work in an anaerobic species (Guiney et al. 1984). To cope with this problem and make a complete shuttle vector, we have constructed a basic plasmid (pCW1) that will accept replication control sequences from native plasmids of rumen bacteria.

Isolation of native plasmids

The first native plasmid, isolated from R. albus, is being prepared at present for insertion into pCW1. This must be-performed by an indirect method because of the problem of direct plasmid cloning mentioned above. It has been necessary, therefore, to insert the plasmid first into a lambda bacteriophage vector, to be grown in E. coli, before insertion into pCW1. Native plasmids have also been identified in isolates of B. fibrisolvens and in a Propionibacterium sp. In all cases, these native plasmids are in the size-range of 2-3.5 kilobase pairs. It will therefore be possible to insert the entire plasmid into pCW1 without increasing the total size unreasonably. Later experiments will be designed to reduce the size of the shuttle vectors, in order to maximise the amount of DNA that can be inserted. The size factor may be a major consideration, if there are a number of genes required to provide efficient cellulolytic activity.

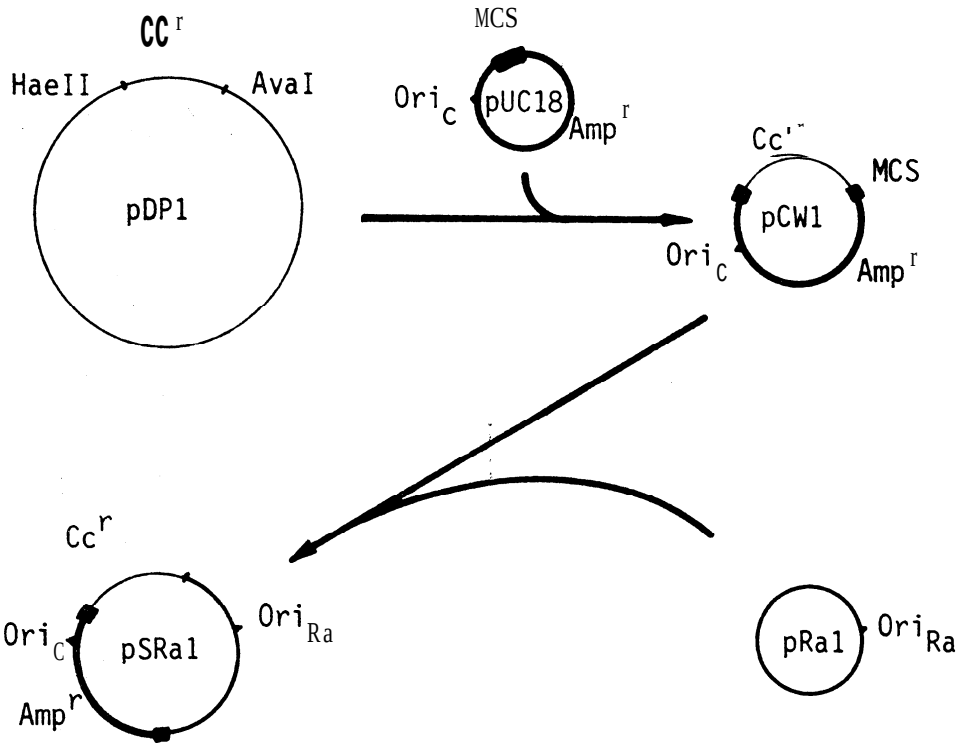


Fig. 2. Pathway for the construction of rumen bacterial shuttle plasmids. The clindamycin resistance gene (Cc^r) has been cloned into the multiple cloning site (MCS) of pUC18. The resulting plasmid (pCW1) therefore contains the origin of replication to allow growth in E. coli (ori_C), Ampicillin resistance gene (Amp^r) as a selectable marker in $coEI^c$ and the Cc^r gene as a selectable marker in anaerobic bacteria. Native plasmids, such as pRa1, will be incorporated to provide suitable replication origins for rumen bacteria ($e.g. ori_{Ra}$).

Transformation of bacteria by electroporation

Several experiments were performed, attempting to introduce the colonic Bacteroides plasmid pDP1 (donated by Dr. J. Brooker) into B. ruminicola brevis, but without success. It is possible that the Bacteroides species from the human colon may be genetically too far removed, from the rumen Bacteroides for their plasmids to be compatible. Experiments in which pDP1 was inserted into E. coli by electroporation succeeded in generating approximately 5000 transformants per microgram of DNA. By conventional standards this is low efficiency and a manufacturer of commercial electroporation devices (Bio-Rad) has claimed efficiencies of 10^6 transformants per microgram of DNA. After preparation of a more suitable plasmid, this method will be tested on rumen bacteria.

CONCLUDING REMARKS

The isolation of genes coding for cellulolytic enzymes and the development of methods for inserting those genes into a wide variety of rumen bacteria, represent only a small part of the work required to achieve the goal of enhancing fibre digestion in vivo. The two technical problems of dominant importance are to engineer the synthesis of fully functional enzymes within recipient bacteria and to ensure that the newly introduced genes do not impose a metabolic burden that will selectively disadvantage the recipient organism. The first problem has been discussed briefly and the solution may involve the need for post-translational modification of enzymes and/or mechanisms to locate the enzyme in the appropriate position on the outer membrane. If so, additional genes may need to be co-transferred to the recipient organism. The second problem of "metabolic burden" is the one most commonly raised by critics of the concept of genetically engineering rumen bacteria, that an altered organism may not compete well in the rumen.

The most likely cause of competitive disadvantage in a modified bacterium, is the production of enzymes in an unregulated manner. Synthesis of cellulase during times of high soluble-nutrient availability would provide an energy drain that could be expected to slow population growth, relative to unmodified organisms. Therefore, it is clear that the enzymes must be produced only when the production will provide an advantage to the engineered bacterium. Fibrolytic enzymes generally are repressed by the presence of simple sugars and/or induced by the presence of complex carbohydrates. Therefore, the mechanisms for regulation of f ibrolytic enzymes already exist. It remains for those controlling factors to be included during the process of introducing new genes. Consequently , the elucidation of control mechanisms, has to be a major part of the characterization of cellulose-digesting enzymes.

So far, one part of this engineering process that has not been mentioned is the need for any introduced genes to be established within the engineered organism as stable, heritable characteristics. Clearly the introduction of plasmids into rumen bacteria does not fulfill this requirement. In some way, the genes introduced on a plasmid must be incorporated into the genome of the recipient bacterium. One possible approach to this probl em may be to subject the altered organism to some form of physical assaul t , such as ult raviolet 1 ight, in order to induce the repair mechanisms of recombinat ion. The inclusion of host-homologous sequences in the shuttle vcct or may be necessary to encourage recombination of plasmid sequences into the genome. Ext ens ive characterization of the resultant organism will be required to ensure that the desired f ibrolytic capabilities and necessary control factors remain functional after such treatment.

During the development of this project we have attempted to consider the major complications and difficulties involved in reaching the final aims. It is vital to stress the long-term nature of the work, which results directly from the many complexities involved. However, there can be little doubt that, in the shorter term, the information acquired in the process will provide a vaulable understanding of rumen micro-organisms and their genetics. It is clear that the genetic manipulation of rumen bacteria holds great potential for increasing the efficiency of animal production. It is equally clear that the realization of that potential can only be achieved through the commitment, to that aim, of a major research effort.

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