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The development of vectors and transfer systems for the rumen bacterium *Butyrivibrio fibrisolvens*

BY

Rhonda G. Clark 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fuifillment of the requirements for the degree of **Doctor of Philosophy in Animal Biotechnology**

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE DEVELOPMENT OF VECTORS AND TRANSFER SYSTEMS FOR THE RUMEN BACTERIUM *BUTYRIVIBRIO FIBRISOLVENS* submitted by RHONDA G. CLARK in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ANIMAL BIOTECHNOLOGY.

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ABSTRACT

This study reports on the cloning of an amylase gene from *Streptococcus bovis* 033 and the development of vectors and associated transfer systems for *Butyrivibrio fibrisolvens.*

Amylase positive clones were identified from a *S. bovis* 033 λgtWESλB genomic library. An amylase insert of 7.5 kb was isolated from one of the clones. Subcloning localized the amylase gene to a region of approximately 3.1 kb. Expression of the amylase gene in *Escherichia coli* subclones was largely associated with the cytoplasmic fraction. Southern hybridization of genomic DNA from the amylolytic strains. *S. bovis* 033, *S. bovis* 077, *B. fibrisolvens* 194 and 195, revealed a single hybridizing band in *S. bovis* 033 DNA only.

Vectors were developed for *B. fibrisolvens* based on pBF194, a cryptic *B. fibrisolvens* plasmid, and other well characterized plasmids from Gram positive and Gram negative bacteria. Using a modified electroporation protocol the *Staphylococcus aureus* plasmid pUB110 and the pUB110-based shuttle vectors, pUBLRS and pLRS07Km, were introduced into *B. fibrisolvens* strain H17c at average transfer frequencies of 2.60×10^1, 3.28×10^2, and 5.00×10^1 CFU/μg, respectively. *B. fibrisolvens* H17c was the only strain transformed using electroporation.

A conjugative transfer system based on Tn*916*-mediated transfer of pUB110 was developed. *B. fibrisolvens* donor strains H17c2 and H17c12, containing Tn*916* and pUB110 or pUBLRS, were constructed. Transfer of pUB110 from H17c2 into strains 193, 194, and 195 occurred at a combined average frequency of 7.78×10^{-7} per

donor and 1.11 x 10^{-5} per recipient. pUBLRS transconjugants of strains 193 and 194 were detected at a combined average frequency of 1.22 x 10^{-6} per donor and 4.70 x 10^{-8} per recipient. The presence of pUB110 and pUBLRS in transconjugants was confirmed by Southern hybridization analysis.

The chloramphenicol resistance gene of the *S. aureus* plasmid pC194 was cloned into pLRS07Km and the amylase gene of *S. bovis* 033 was cloned into pUBLRS. These recombinant vectors, pLRS07Km/Cm and pUBLRSA, were introduced into strain H17c by electroporation. H17c transconjugants containing pLRS07Km/Cm were resistant to 5 µg/ml of chloramphenicol. Zymogram analysis of pUBLRSA transformants revealed novel bands of amylase activity in addition to those of H17c.

In this study vectors were developed which could be transferred into *B. fibrisolvens* using electroporation or conjugation. These systems constitute a significant contribution to genetic studies in this organism.

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1.0 INTRODUCTION

Attempts to manipulate ruminant digestion via conventional approaches in order to improve the efficiency of feed conversion have met with limited success. Most studies have concentrated on the treatment of feeds to enhance or alter some aspect of digestion. In such a complex ecological system as the rumen it may actually be more beneficial to alter the composition of the plant material, through plant breeding, or to alter the metabolic capability of select rumen microorganisms (Forsberg and Cheng, 1992). Genetically modified rumen bacteria could be beneficial to the ruminant and may improve certain aspects of ruminant digestion (Smith and Hespell, 1983; Hazlewood and Teather, 1988; Russell and Wilson, 1988; Gilbert and Hazlewood, 1991).

In order to study rumen microorganisms at a genetic level there are three major requirements: (1) the cloning of genes encoding enzymes involved in plant polysaccharide digestion, (2) development of shuttle vectors specific for rumen bacteria to re-introduce these cloned genes and study their regulation and expression in rumen bacteria, (3) development of transfer systems to introduce the shuttle vectors into the rumen bacteria.

Most molecular biology studies of rumen microorganisms have involved the isolation of bacterial and fungal genes encoding for enzymes involved in the degradation of cellulose and hemicellulose. The expression and regulation of these genes has been studied in *Escherichia coli* and many of these genes are now being sequenced (Berger et al., 1990; Lin et al., 1990). The analysis of the regulation

of these genes in rumen bacteria requires shuttle vectors and transfer systems specific for these microorganisms. Presently, there are few useful vectors and methods of transfer that can be used to reintroduce genes into rumen bacteria.

Two rumen bacteria, *Streptococcus bovis* and *Butyrivibrio fibrisolvens,* were used in this study. *S. bovis* is important in the degradation of starch and has been implicated in the development of conditions such as lactic acidosis and bloat in animals on high grain diets. Isolation of amylase genes from *S. bovis* would facilitate the study of the numbers of genes involved in starch degradation and how they are regulated. *B. fibrisolvens* is one of the predominant bacteria isolated from the rumen and members of this genus are involved in the digestion of starch, hemicellulose, cellulose, lipids, proteins, and pectin. This metabolic diversity enables *B. fibrisolvens* to be present in the rumen at significant levels under varied feeding conditions. The stability of *B. fibrisolvens* in the rumen indicates that it would be a suitable bacterium for genetic modification.

The objectives of this study were:

1. To clone an amylase gene from *S. bovis* and study its expression in *E. coli.*
2. To develop vectors capable of replicating in *B. fibrisolvens* into which genes, such as the *S. bovis* amylase, could be cloned, and
3. To develop transfer systems for *B. fibrisolvens* based on electroporation or conjugation which could be used to introduce the developed shuttle vectors into this bacterium.

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2.0 LITERATURE REVIEW

2.1 The rumen environment

Herbivorous mammals have developed a symbiotic association with microorganisms in their gut which enables them to digest plant structural polysaccharides. The stomach of ruminants has four compartments which include the rumen, reticulum, omasum, and the abomasum. In the adult ruminant, the rumen is the largest of these four compartments, comprising approximately 80% of the total stomach volume. The majority of plant polysaccharide digestion occurs within the rumen.

The environment of the rumen is highly suitable for the growth of rumen microorganisms. Under normal conditions the rumen is maintained at a temperature of 39°C (Hungate, 1966). Rumen fluid pH ranges from 5.9 to 7.0 and is buffered by bicarbonate contained in the saliva (Counotte et al., 1979). The rumen is anaerobic with a redox potential ranging from -250 to -450 mV (Church, 1975).

Four interrelated compartments have been identified in the rumen and these consist of a liquid phase, a solid mass of digesta, a gas phase, and the rumen epithelium. The gaseous phase contains numerous gases but consists primarily of carbon dioxide (65%) and methane (35%) (Hobson and Wallace, 1982). Inflation of the rumen with gas stimulates the eructation reflex which results in the release of some of these gases. When the cardia region of the reticulum, at the oesophageal opening is covered with ingesta, water, or foam, a condition known as bloat can occur. An example of this is pasture bloat where the release of large amounts of soluble

nutrients from legume forage materials causes the formation of frothy foam that traps the gases in the rumen (Howarth et al., 1986).

Movement of plant material into the rumen begins with chewing and mixing of the feed with saliva and formation into a bolus. This bolus is swallowed and carried to the rumen via peristaltic contractions of the oesophagus. Contractions of the rumen mix the bolus with the rumen contents and brings the plant material in contact with the rumen microorganisms which begin digestion of the plant material. Rumination, which is the regurgitation of a bolus containing larger plant fragments and subsequent re-chewing, is stimulated by larger plant materials in the reticulo-rumen. Rumination and microbial digestion of plant materials results in the gradual decrease in particle size and an increase in particle density (Orpin, 1982). The denser particles move to the reticulum where they are passed into the omasum and finally into the abomasum where digestion of the residual particles and the microorganisms occurs. Screening of large particles and absorption of water, acids and other substances occurs in the omasum while the abomasum functions the same as in monogastric animals.

2.2 The role of rumen microorganisms in digestion

The rumen microorganisms, which include protozoa, fungi, and bacteria, facilitate the digestion of plant material under anaerobic conditions providing energy and protein to the ruminant animal. The protozoa play a role in fiber and protein digestion but generally are

not considered essential in the digestion of feeds (Hungate, 1966). Rumen fungi play an important role in fiber digestion in the rumen and are primary invaders of plant material helping to make these materials more accessible to bacteria (Li and Heath, 1993). The fungi attach to lignified vascular tissues of plants, which bacteria are incapable of digesting, suggesting a unique activity for the rumen fungi (Akin, 1986). The rumen bacteria play a major role in ruminant digestion. Certain genera are predominant in fiber digestion, such as *Fibrobacter succinogenes, Ruminococcus albus,* and *R. flavefaciens,* while other genera digest other plant polysaccharides or utilize endproducts of primary digestion. The predominant fibrolytic rumen bacterium of ruminants consuming low quality forage is *Butyrivibrio fibrisolvens* (Orpin et al., 1985).

It is evident that the microbial population of the rumen is heterogenous with each organism occupying a particular niche within this complex ecosystem. The numerous interactions between the microorganisms results in a relatively efficient digestive process.

2.3 Compartmentation of the microbial population within the rumen

The rumen is highly structured and its microbial population can be divided into four interrelated phases which have been described by Czerkawski and Cheng (1988). The first of these phases contains the microorganisms associated with the rumen liquid. Bacteria in this phase are not numerically important but

could act in detoxification of toxic plant compounds (Majak and Cheng, 1981) and as an inoculum for ingested plant materials.

Phase 2 has been labelled as the shuttle phase. The bacterial population of this phase is closely associated with those in phase 3, the particle-associated population. This particle-associated population has been shown to constitute 75% of the rumen microbial population (Forsberg and Lam, 1977). Bacteria of phase 2 transfer and utilize microbial nutrients and endproducts of metabolism from the primary digesters of phase 3.

Phase 4 incorporates bacteria associated with the rumen wall, which account for only 1% of the microbial population of the rumen. These bacteria are generally facultative anaerobes which scavenge any oxygen present. This population also carries out proteolytic digestion and recycling of sloughed rumen epithelial cells as well as the conversion of urea, secreted through the rumen wall, to ammonia which is used in bacterial nitrogen metabolism.

2.4 Bacterial attachment to feed and the establishment of multi-species consortia

Once the plant material is in the rumen, contractions of the rumen mix it with the rumen liquid thereby beginning the establishment of multi-species consortia. Numerous bacteria, each with certain metabolic abilities will work together to degrade cellulose, hemicellulose, proteins, starch, pectins, and lipids contained in the plant material. The rumen fungi also play a role in digestion, primarily in fiber degradation. Rumen fungi are the

primary invaders of plant fibers and together with chewing by the ruminant they make the plant material more accessible to the bacteria (Li and Heath, 1993).

Digestion of the plant material proceeds in an "inside-out" fashion with the more readily degradable internal tissues being digested completely and the more recalcitrant outer components being partially digested (Cheng et al., 1991). Microbial attachment to fibrous plant material has been demonstrated and is known to be essential for degradation of plant fiber (Kudo et al., 1987b). Capsular structures of some fibrolytic bacteria are responsible for attachment of bacteria to fibrous plant materials (Cheng et al., 1984). Chemically different plant cell walls are colonized by different cellulolytic bacteria (Cheng et al., 1984). For example, pectin-rich walls are preferentially colonized by *Ruminococcus* and *Lachnospira*. *R. flavefaciens* attaches to cellulosic grass walls while *F. succinogenes* tends to attach to more recalcitrant structures such as straw cell walls.

The complexity of the plant material means that the activity of a single microorganism will not result in its digestion. In this ecological system two or more species combine their enzymatic activities to degrade these complex plant tissues. The bacteria involved in digestion will form multi-species consortia. Once adherent bacteria (phase 3) are established other bacteria will assemble in close proximity (phase 2). The adherent population provides nutrients to the closely associated population through the primary digestion of the plant structural polysaccharides and

together these microbial activities drive the digestive process. Evidence for these associations can be seen in laboratory studies comparing cellulose degradation of pure cultures and mixed cultures. The rate and extent of cellulose digestion in cultures containing a mixture of *F. succinogenes* and *Treponema bryantii* approaches that seen in the rumen (Kudo et al., 1987a). This same effect can also be seen with combinations of rumen fungi and methanogenic bacteria (Wood et al., 1986). Alternatively, bacterial associations with fungi can decrease cellulolytic activity and this has been speculated to be the reason why fungi are usually associated with the more recalcitrant plant materials which bacteria do not colonize (Forsberg and Cheng, 1990). Overall, digestion of plant material in the rumen is enhanced due to microbial attachment and the development of compatible consortia that act together to digest plant components.

2.5 The nature of plant materials and their degradation

Ruminant diets normally consist of cereal grains and forages. Forages are predominant in ruminant diets with small amounts of grains fed in certain situations. Feeding of a diet consisting of cereal grains leads to the establishment of amylolytic microorganisms whereas diets high in forage result in a population of fibrolytic microorganisms.

Cereal grains are composed primarily of starches with a small percentage of cell wall material consisting of cellulose, hemicellulose, and lignin (Forsberg and Cheng, 1990). The intact

structure of cereal grains is highly resistant to microbial penetration because of the presence of a waxy cuticle (Cheng et al., 1991). The processing of cereal grains such as rolling, together with chewing by the animal, makes the components of the cereal grain accessible to microorganisms. Attachment of amylolytic bacteria like *Streptococcus bovis, Prevotella ruminicola,* and *Butyrivibrio fibrisolvens* to the grain results in digestion (McAllister et al., 1990). The starch in cereal grains is rapidly hydrolyzed by cell associated or extracellular amylases, presumably α-amylase, to maltooligosaccharides and glucose (Cotta, 1988). Preliminary work has indicated that the maltooligosaccharides produced may be used by other non-amylolytic bacteria, resulting in crossfeeding (Cotta, 1992). The predominant microbial endproduct of starch digestion is lactate, which is utilized primarily by *Megasphaera elsdenii.*

The cell walls of forages are composed primarily (40-68%) of a matrix of cellulose and hemicellulose linked to varying degrees to lignin (Forsberg and Cheng, 1990). The anatomy of the plant as well as the organization and composition of the plant cell wall constituents influences microbial digestion of forages (Akin, 1986). Cellulose is composed of β–1,4-linked glucose molecules while hemicellulose is composed mainly of xylan formed from β–1,4-linked xylose residues (Forsberg and Cheng, 1990).

Cellulases and xylanases, which are produced by cellulolytic rumen bacteria and fungi, encompass a collection of enzymes whose primary function is to hydrolyze the β–1,4-glycosidic linkages in

cellulose and hemicellulose (Gilbert and Hazlewood, 1993). The broad range of cellulases and xylanases required for fiber digestion is outlined in figure 1 (Forsberg et al., 1993). The cellulolytic fungi and bacteria contain a range of these enzymes. In *Fibrobacter succinogenes* numerous cellulases have been purified and many of the genes coding for them have been cloned (Forsberg and Cheng, 1990).

The cellulases hydrolyze bonds in the amorphous regions of cellulose (endoglucanases) which creates non-reducing ends from which cellobiose is released by cellobiosidases. The cellobiose is then cleaved by cellobiases into glucose which is used by the microorganisms. Organisms such as *R. albus* and *R. flavefaciens* can metabolize cellobiose. Unlike cellulose, xylan, the major component of hemicellulose, requires an array of enzymes for digestion. As seen in figure 1, the xylan backbone is highly substituted. The battery of enzymes listed hydrolyze xylan into its constituent sugar, xylose. Xylose is utilized by microorganisms via the pentose phosphate pathway.

Lignin is a polymer made up of many phenylpropanoid units associated in a complex cross-linked molecule (McDonald et al., 1985). There are no rumen microorganisms that can degrade lignin although rumen fungi attach to lignin. This structural component is therefore inaccessible and is of no nutritive value to the microorganisms or the ruminant animal.

Another carbohydrate of plant cell walls available to the rumen microorganisms is pectin. Pectin is usually found in the

middle lamella of plants and in the primary cell wall (Clarke and Bauchop, 1977). D-galacturonic acid is the main constituent of this polysaccharide (McDonald et al., 1985). Pectinases are produced by rumen microorganisms such as *F. succinogenes, P. ruminicola, B. fibrisolvens, and L. multiparus,* and degrade this plant cell wall carbohydrate into galacturonic acids which then are degraded to yield xylose (Clarke and Bauchop, 1977).

Protein degradation is due to numerous microbial activities including protein hydrolysis, peptide degradation, amino acid deamination, and fermentation of the resulting carbon skeletons (Cotta and Hespell, 1986). Most of the ingested protein is degraded by the rumen microorganisms and the majority of protein used by the animal is microbial in origin. Seventy-five percent of proteolytic activity is associated with the solid-associated microbial population and the proteolytic enzymes of the bacteria are predominantly cell-associated (Cotta and Hespell, 1986). A large proportion of the rumen bacteria are proteolytic (eg. *Prevotella ruminicola, Ruminobacter amylophilus, Butyrivibrio fibrisolvens, Streptococcus bovis, Selenomonas ruminantium, Lachnospira multiparus*) and their combined activity contributes to protein degradation (Russell and Wallace, 1988). The rumen fungus *Neocallamastix frontalis* is also highly proteolytic and contributes to protein degradation.

The endproduct of protein degradation is primarily ammonia, with minor amounts of small peptides and free amino acids also being produced. These products are utilized by the rumen

microorganisms to sythesize microbial proteins. Branched-chain fatty acids found in rumen liquid are derived from the deamination of certain amino acids (eg. valine is deaminated to form isobutyric acid).

Lipids, contained in low quantities in feed, do not represent a significant energy source for the microorganisms but rather are primarily used for synthetic purposes. Rumen bacterial lipases hydrolyze lipids to release them from ester combinations. Glycerol is fermented by microorganisms and unsaturated fatty acids are hydrogenated by the bacteria (McDonald et al., 1985).

2.6 Use of endproducts of digestion

The endproducts of plant polysaccharide degradation are utilized by the rumen microorganisms to synthesize volatile fatty acids (VFA's) and microbial protein which are used by the ruminant as energy and protein sources, respectively. The major volatile fatty acids produced by the microorganisms are acetate, propionate, and butyrate. Small amounts of lactate, succinate, and formate are produced but these are generally metabolized by other rumen bacteria within the digestive consortia. Carbon dioxide and hydrogen are also endproducts of bacterial metabolism. These gases are used by methanogens to produce methane, which comprises a large percentage of the gas phase in the rumen.

Acetate is absorbed by the reticulo-rumen in the largest amount and is the highest circulating VFA in the bloodstream (Van Soest, 1982). It is a lipogenic precursor and is used in fatty acid

synthesis, through carboxylation to malonyl-CoA. Butyrate is partly metabolized by the rumen epithelium to ketone bodies which are converted to fats in the liver of the animal (Van Soest, 1982). Propionate is used by the animal in gluconeogenesis. This VFA enters the citric acid cycle, through a series of steps, as succinate and from this point can be converted to glucose; form citrate through condensation with acetyl-CoA; or be converted to aspartate, alanine, or glutamate through reductive amination (Van Soest, 1982).

The other VFA's produced are utilized by various rumen bacteria. Lactate is used predominantly by *Megasphaera elsdenii,* which utilizes about 74% of the lactate produced in the rumen, and to some extent by *Selenomonas ruminantium* (Russell and Wallace, 1988). The fate of this VFA is its conversion to propionate via the non-randomizing (acrylate) pathway (Van Soest, 1982). Succinate produced by bacteria like *P. ruminicola,* and *R. amylophilus* does not accumulate in the rumen as it is converted to propionate (Clarke and Bauchop, 1977). Formate is broken down into carbon dioxide and hydrogen via the enzyme, formate-H_2 lyase, and these gases are added to the gas phase or used by methanogens (Russell and Wallace, 1988).

Anaerobic metabolism means that ATP must be generated from anaerobic reactions. Ruminant digestion is very effective in converting fibrous feeds into substrates that can be utilized by the animal for energy, namely the VFA's. The microorganisms themselves will, once they are washed from the rumen to the abomasum, become a source of protein for the ruminant. This

symbiotic association allows ruminants to utilize low quality feeds to produce a high quality product.

2.7 Applying recombinant DNA techniques to rumen microorganisms

2.7.1 Gene cloning and shuttle vectors

Progress has been made in the area of gene cloning. Most of the genes cloned from rumen microorganisms have been cellulases and xylanases due to the fact that these enzymes convert cellulose and xylan, the major plant structural polysaccharides, to their constituent sugars. Since forages comprise a large part of the diet of ruminants these enzymes are central to ruminant digestion. Additionally, these enzymes are of commercial interest since they are capable of converting plant biomass into renewable substrates for the chemical, pharmaceutical, and feed industries (Gilbert and Hazlewood, 1993).

Very few vectors and methods of transferring these vectors have been developed for any of the rumen bacteria being studied. Recently, there has been developments in this area which will be discussed, together with genes cloned, in the following sections.

2.7.1.1 Rumen fungi. Gene cloning and enzyme purification studies of some of the rumen fungi has revealed some interesting features of these polysaccharidases. The xynA gene cloned from *N. frontalis* expresses a bifunctional xylanase, XYLA, which has the same xylanase activities in its N-terminal and C-terminal regions suggesting there has been a tandem duplication of the xynA gene

(Gilbert et al., 1992). What is most interesting though is that this fungal enzyme shares homology with a *Ruminococcus flavefaciens* polysaccharidase, which seems to indicate the possibility of horizontal gene transfer in the rumen (Gilbert et al., 1992). Two different bifunctional β-endoxylanase enzymes were purified from *N. frontalis* which exhibited endoglucanase and xylanase activity (Gomez de Segura and Fevre, 1993). A *N. patriciarum* cDNA clone, celD, was shown to encode for a multifunctional enzyme which expressed endoglucanase, cellobiohydrolase, and xylanase activity (Xue et al., 1992a). This same research group has cloned three other endoglucanase genes, celA, celB, and celC, from this fungus (Xue, 1992b). These three genes were shown to be induced by cellulose while celD was produced constitutively (Xue et al., 1992a & b). A xylanase of *N. patriciarum* 27 exhibited activity on carboxymethylcellulose (CMC) and had the ability to bind to cellulose (Tamblyn Lee et al., 1993). These few studies show the organizational complexity of the polysaccharidases in rumen fungi. In the rumen, multifunctional enzymes encoded by one gene could provide an advantage to an organism for utilizing the many components of forages.

2.7.1.2 ***Streptococcus bovis.*** Numbers of *S. bovis* have been shown to increase in the rumen of animals switched from hay diets to high grain diets. The emphasis of genetic experiments has been the cloning and isolation of amylases from this organism. An amylase gene has been cloned from *S. bovis* strain 033 into *E. coli* (Clark et al., 1992). An extracellular amylase of *S. bovis* JB1 was

cloned and studied in *E. coli* and was then re-introduced into JB1, by electroporation, using the *Streptococcus sanguis/E. coli* shuttle vector pVA838 (Whitehead, 1992; Cotta and Whitehead, 1993). Two different α-amylases cloned from *S. bovis* 148 have been extensively characterized (Satoh et al., 1993). The first amylase, AmyI, is an extracellular enzyme while AmyII apparently is an intracellular amylase (Satoh et al., 1993).

Besides the use of electroporation for transformation of *S. bovis,* as mentioned above, there has also been the development of a conjugative system of transfer. The conjugative tetracycline resistant transposon Tn*916* can be transferred from *Enterococcus faecalis* into *S. bovis* JB1 at a frequency of 6.9 x 10^{-6} per recipient (Hespell and Whitehead, 1991). A conjugative plasmid, pAMβ1, was also transferred from *E. faecalis* into *S. bovis* JB1 (Hespell and Whitehead, 1991). Strain JB1 could subsequently donate pAMβ1 back to *E. faecalis* and also to *Bacillus subtilis* and three strains of *B. fibrisolvens* (Hespell and Whitehead, 1991). These systems should prove useful for gene transfer in *S. bovis.*

2.7.1.3 *Selenomonas ruminantium.* *S. ruminantium* is also involved in starch digestion and some strains are also involved in the fermentation of glycerol and lactate (Hungate, 1966; Hespell and Bryant, 1981). Most genetic work in *S. ruminantium* has focused on the isolation of bacteriophage and cryptic plasmids and there have been no genes cloned from this bacterium. Temperate bacteriophage have been isolated that are capable of infecting *S. ruminantium* strains M-7 and HD4 (Lockington et al., 1988). The bacteriophage

could also be introduced into these strains using electroporation and PEG-mediated transformation (Lockington et al., 1988).

A 4.8 kb cryptic plasmid, pSR1, identified in *S. ruminantium* HD4, was characterized by restriction mapping (Dean et al., 1989; Martin and Dean, 1989). It was observed that certain enzymes cut this plasmid only when it was propagated in *E. coli,* suggesting the presence of a restriction-modification system in this bacterium. Two plasmids from *S. ruminantium* subsp. *lactilytica,* pJDB21 and pJDB23, have been completely sequenced (Attwood and Brooker, 1992; Zhang and Brooker, 1993). Both of these plasmids replicate in *E. coli,* with pJDB21 replicating via a single-stranded intermediate which is common to gram positive plasmids such as pUB110 (Zhang and Brooker, 1993). Cryptic plasmids, approximately 2.5 kb in size (Murray et al., 1992), have been identified in two other *S. ruminantium* strains, FB315 and 521C1.

These isolated plasmids will be useful in the construction of shuttle vectors for *S. ruminantium.* Shuttle vectors have been constructed based on pS23, from *S. ruminantium* S23, and the *E. coli* vector pUC19, in addition to additional antibiotic resistance genes (Brooker et al., 1990). These constructs, pUCS23, pS23CAT, and pS23tet could not be introduced into *S. ruminantium* (Brooker et al., 1990). A mobilization region from a conjugative plasmid was cloned into pS23tet and attempts to mobilize this construct from the *E. coli* donor strain S17.1 into *S. ruminantium* failed, although it could be mobilized into *E. coli* DH1 (Brooker et al., 1990). As noted earlier, there could be restriction barriers present that hinder the ability to

introduce shuttle vectors into *S. ruminantium.* Additionally, vectors constructed thus far have been based on gram negative plasmids and it is possible that constructs based on gram positive plasmids may prove more successful.

2.7.1.4 *Prevotella ruminicola.* *P. ruminicola,* formerly known as *Bacteroides ruminicola,* was reclassified based on recent taxonomic studies (Shah and Collins, 1990). *P. ruminicola* strains are involved in the degradation of starch, protein, pectin, and hemicellulose (Hungate, 1966). The genes cloned from this organism include endoglucanases and xylanases (Whitehead and Hespell, 1989; Matsushita et al., 1990; Matsushita et al., 1991; Whitehead and Lee, 1991; Whitehead, 1993). The cloned endoglucanase from *P. ruminicola* B_14 has sequence homology with regions of sequence from β-glucanases of *R. albus* and *C. thermocellum* (Matsushita et al., 1990). The enzyme produced from a cloned *P. ruminicola* 23 xylanase gene had homology with the catalytic domain of endoglucanases from *B. fibrisolvens, R. flavefaciens,* and *C. thermocellum* (Whitehead, 1993). A survey of the distribution of xylanase genes among 26 strains of *P. ruminicola* was done using a xylanase/endoglucanase gene from strain 23 and a xylanase gene from strain D31d (Avgustin et al., 1992). This study revealed that not all of the strains had homologous xylanases and that strains whose DNA hybridized to the genes exhibited variations in the molecular masses of the xylanases when zymogram analysis was done.

Genetic experiments of cloned polysaccharidases of *P. ruminicola* have progressed to studying transcriptional regulation

and constructing hybrid enzymes. The regulation of transcription of the *P. ruminicola* AR20 endoglucanase was studied in *E. coli* and consensus sequences were found that were similar to those of *E. coli* (Vercoe and Gregg, 1992). A reconstructed endoglucanase, which joined an endoglucanase of *P. ruminicola* to the cellulose binding domain (CBD) of *Thermomonospora fusca,* exhibited more activity than the original *P. ruminicola* endoglucanase (Maglione et al., 1992). Some interesting work has also been done with the xylanase gene from *P. ruminicola* 23. This xylanase was cloned into pVAL-1, a colonic *Bacteroides* vector, and transferred by conjugation into *B. fragilis* and *B. uniformis* where it exhibited activity 1400 times greater than in *P. ruminicola* (Whitehead and Hespell, 1990). The xylanase-pVAL-1 plasmid was not stable in the absence of antibiotic selection so this gene was incorporated into the chromosome of *B. thetaiotaomicron* (Whitehead et al., 1991). This chromosomal xylanase gene was stable which meant that this recombinant organism could potentially be introduced into the rumen for study (Whitehead et al., 1991).

Conjugative systems for introducing plasmids into *P. ruminicola* have been established. One of the first demonstrations of conjugative transfer in *P. ruminicola* involved the transfer of a tetracycline resistant plasmid, identified in *P. ruminicola* strains 23 and 23/M2/7, into other *P. ruminicola* strains (Flint and Stewart, 1987; Flint et al., 1988). Other plasmids identified in strains 23 and 23/M2/7 have subsequently been used to construct conjugative shuttle vectors that were mobilized from colonic *Bacteroides* into *P.*

ruminicola NCFB 2202 (Thomson et al., 1992; Bechet et al., 1993). pRDB5, a vector based on a colonic *Bacteroides* plasmid and tetracyline resistance gene, was mobilized from *B. uniformis* into *P. ruminicola* (Shoemaker et al., 1991). The tetracycline gene used is from a chromosomal element, TcrEmr12256, originally identified in *B. fragilis.* This chromosomal element could transfer itself from *B. uniformis* into *P. ruminicola* (Shoemaker et al., 1992). The tetracyline gene of TcrEmr12256 was found to be homologous to the tetracyline gene found on pRRI4, a conjugative plasmid from *P. ruminicola* 223/M2/7 (Flint et al., 1988), suggesting that horizontal transfer has occurred between colonic *Bacteroides* and ruminal *Prevotella* strains (Shoemaker et al., 1992).

An electroporation protocol developed for *P. ruminicola* has been used to introduce various plasmids and vectors into some, but not all, strains of *P. ruminicola* (Thomson and Flint, 1989; Bechet et al., 1993). Transfer systems for *P. ruminicola* have proven successful but it is interesting to note that there are limitations to the applicability of one protocol, whether it is using electroporation or conjugation, to all strains of *P. ruminicola.*

2.7.1.5 *Fibrobacter* (*Bacteroides*) *succinogenes.* *F. succinogenes* is the predominant fibrolytic bacterium of the rumen (Hungate, 1966). The cloning of polysaccharidases in *E. coli* has helped elucidate the numbers of cellulases present in *F. succinogenes.* Eight endoglucanases in total have been cloned from *F. succinogenes* S85 (Forsberg et al., 1993). *cel*3, the most characterized of these genes, codes for a protein that exhibits both

endoglucanase and cellobiohydrolase activity and has homology with *celC* from *C. thermocellum* (McGavin et al., 1989). A recently isolated carboxymethylcellulase (endoglucanase) from *F. succinogenes* S85 was found in a number of *F. succinogenes* strains tested using Southern hybridization analysis (Forano et al., 1994). This endoglucanase was not similar to any of the eight previously isolated genes and it is possible that it codes for the endoglucanase EG2 which was not represented in the original eight clones (Forsberg et al., 1993). A cellodextrinase has also been cloned from S85 which did not possess endoglucanase activity (Gong et al., 1989). A mixed linkage β-glucanase of S85, specific for glucans with β-1,3 and β-1,4 linkages, has been sequenced and does not exhibit any homology to *cel3* (Irvin and Teather, 1988; Teather and Erfle, 1990). A multifunctional endoglucanase, *end*A_{FS}, from strain AR1 has been cloned (Cavicchioli et al., 1991).

Four unrelated xylanase genes have been cloned from *F. succinogenes* S85 (Forsberg et al., 1993). One of these, pBX1, expressed a xylanase in *E. coli* that was not affected by catabolite repression and had properties similar to the enzyme in *F. succinogenes* (Sipat et al., 1987). A xylanase has also been cloned from strain 135, an Asian water buffalo isolate (Hu et al., 1991).

No vectors or transfer systems have been developed for *F. succinogenes.* The presence of a restriction-modification system and a non-specific endonuclease, DNaseA, in *F. succinogenes* S85 seems to indicate that there are formidable barriers to transformation of this organism (Lee et al., 1992).

2.7.1.6 *Ruminococcus flavefaciens* and *Ruminococcus albus*. *R. flavefaciens* and *R. albus* strains are involved in cellulose and hemicellulose degradation in the rumen (Hungate, 1966). The endproducts produced by this hydrolysis vary with *R. albus* producing mostly ethanol, while *R. flavefaciens* produces succinate, acetate, and formate (Hungate, 1966). Various cellulases and xylanases have been cloned from these bacteria and studied in *E. coli*. A gene cloned from *R. flavefaciens* FD-1, coding for a protein with endoglucanase and endoxylanase acitivity, was induced when cellulose and cellotriose were added to *E. coli* cells containing this clone (Howard and White, 1990; Wang et al., 1993). A cellodextrinase, celA, of FD-1 had no homology to other cellulases according to sequence data of this gene (Barros and Thomson, 1987; Wang and Thomson, 1990). This cellulase was determined to be cell-associated and cleaved cellodextrins to cellobiose, which was a competitive inhibitor of CelA (Brown et al., 1993).

Four xylanase genes of *R. flavefaciens* 17 have been cloned which had four separate chromosomal locations in strain 17 (Flint et al., 1989). Two of these xylanases, XYLA and XYLD, have been characterized more extensively (Zhang and Flint, 1992; Flint et al., 1993). XYLA has two active xylanase regions which released different endproducts upon hydrolysis of oat-spelt xylan (Zhang and Flint, 1992). XYLD exhibited xylanase and glucanase activity on the same polypeptide (Flint et al., 1993). The amino terminus of XYLD was shown to exhibit homology with the corresponding regions of XYLA. The functional significance of these bifunctional enzymes is

unclear.

β-1,4-endoglucanases have been cloned from a number of *R. albus* strains. Two endoglucanases have been cloned from strain F-40 and one of these, Eg1, has been sequenced (Kawai et al., 1987; Ohmiya et al., 1989). Gene truncation of Eg1 has demonstrated regions affecting enzyme stability and optimum enzyme activity (Ohmiya et al., 1991). Two different endoglucanases have been isolated from *R. albus* strains AR67 and AR68 (Ware et al., 1989). Two endoglucanase genes of *R. albus* SY3, celA and celB, have also been cloned into *E. coli* and are apparently not multi-domain cellulases (Poole et al., 1990). Another endoglucanase and a xylanase have also been cloned from strain SY3 (Romaneic et al., 1989). β-glucosidases have been cloned, and some sequenced, from *R. albus* F-40 (Ohmiya et al., 1990b; Deguchi et al., 1991; Takano et al., 1992). A comprehensive cloning experiment isolated ten different cellulase-encoding fragments from *R. albus* 8 which exhibited β-glucosidase, cellobiosidase, and β-1,4-endoglucanase activities (Howard and White, 1988).

As with most of the other rumen bacteria being studied, there is a lack of shuttle vectors and useful transfer systems for *R. albus* and *R. flavefaciens.* Intergeneric protoplast fusion between *R. albus* and a *Fusobacterium varium-Enterococcus faecium* anaerobic recombinant, FE7, resulted in fusants that expressed activities found in both parental strains (Chen et al., 1988). An *R. albus* β-glucosidase was used as a probe to verify the presence of this enzyme in the chromosome of the recombinant (Chen et al., 1988).

Electroporation has been used recently to introduce lactic acid bacterial vectors pPSC22 and pCK17 into fresh isolates of *R. albus* and type strain ATCC 27210 (Cocconcelli et al., 1992). The efficiency was increased to approximately 3 x 10^5 transformants per μg of pPSC22 DNA. Restriction-modification systems have been identified in strains of *R. albus* and *R. flavefaciens* which could make it difficult to achieve higher transformation frequencies with these organisms (Morrison et al., 1992a & b).

A number of *R. albus* and *R. flavefaciens* strains have been screened for the presence of plasmids which could be used in constructing shuttle vectors for these bacteria (Asmundson and Kelly, 1987; Champion et al., 1988). Two plasmids of *R. albus* F-40, pRAB and pRAC, may code for cellulose utilization functions (Ohmiya et al., 1990a). It would be interesting to see these plasmids probed with some of the cloned cellulases, mentioned earlier, from F-40. Deletion studies of these plasmids and re-introduction into *R. albus* could also provide information on their function.

2.7.1.7 *Butyrivibrio fibrisolvens.* *B. fibrisolvens* can be isolated from a variety of animals in many geographical areas (Hespell and Bryant, 1981). Members of this genus are very versatile with most strains digesting hemicellulose, pectin, protein, and starch, while some strains are capable of digesting cellulose (Hespell and Bryant, 1981). The major endproduct of *B. fibrisolvens* fermentation is butyrate (Hungate, 1966).

Cellulases have been cloned from *B. fibrisolvens* although this organism is not known to be involved to any great extent in cellulose

degradation. An exception is in animals being fed low quality forages, where *B. fibrisolvens* is the predominant cellulolytic bacterium (Margherita and Hungate, 1963; Orpin et al., 1985). A β-glucosidase has been cloned from *B. fibrisolvens* H17c (Lin et al., 1990). A cellodextrinase gene cloned into *E. coli* coded for an enzyme which hydrolyzed cellodextrins to cellobiose or cellobiose and glucose (Berger et al., 1990). Endoglucanases from strains A46 and H17c have been cloned and sequenced (Berger et al., 1989; Hazlewood et al., 1990).

A xylanase from *B. fibrisolvens* 49 was present in only two of five *B. fibrisolvens* strains analyzed by Southern hybridization (Mannarelli et al., 1990). A bifunctional enzyme of strain GS113, coded for by a single gene (*xylB*), exhibited β-D-xylosidase and α-L-arabinofuranosidase activity (Sewell et al., 1989; Utt et al., 1991). A xylanase gene has also been cloned from strain H17c and sequenced (Lin and Thomson, 1991).

B. fibrisolvens is also involved in the hydrolysis of starch. An α-amylase has been cloned from H17c and study of the enzyme revealed that major endproducts from starch hydrolysis were maltose and glucose (Rumbak et al., 1991a). A gene, *glgB,* isolated from H17c coded for an enzyme that produced clearing on starch azure plates (Rumbak et al., 1991b). This enzyme did not display hydrolytic activity on soluble starch but was determined to be a glycogen-branching enzyme.

Plasmids have been isolated from strains of *B. fibrisolvens.* Some of these have been sequenced and some have been used in the

construction of vectors (Teather, 1982; Mann et al., 1986; Hefford et al., 1993). Only one group has been successful in introducing a constructed shuttle vector, containing a *B. fibrisolvens* plasmid, back into *B. fibrisolvens* but the transfer frequency was very low (Ware et al., 1992). Additionally, the antibiotic resistance gene of the vector was not expressed very well in *B. fibrisolvens* (Ware et al., 1992). Whitehead (1992) has used electroporation to introduce a *Bacillus subtilis/E. coli* shuttle vector into *B. fibrisolvens* H17c. Using conjugation a conjugative plasmid, pAMβ1, and a tetracycline resistant conjugative transposon, Tn*916*, have been transferred from *Enterococcus faecalis* into *B. fibrisolvens* (Hespell and Whitehead, 1991).

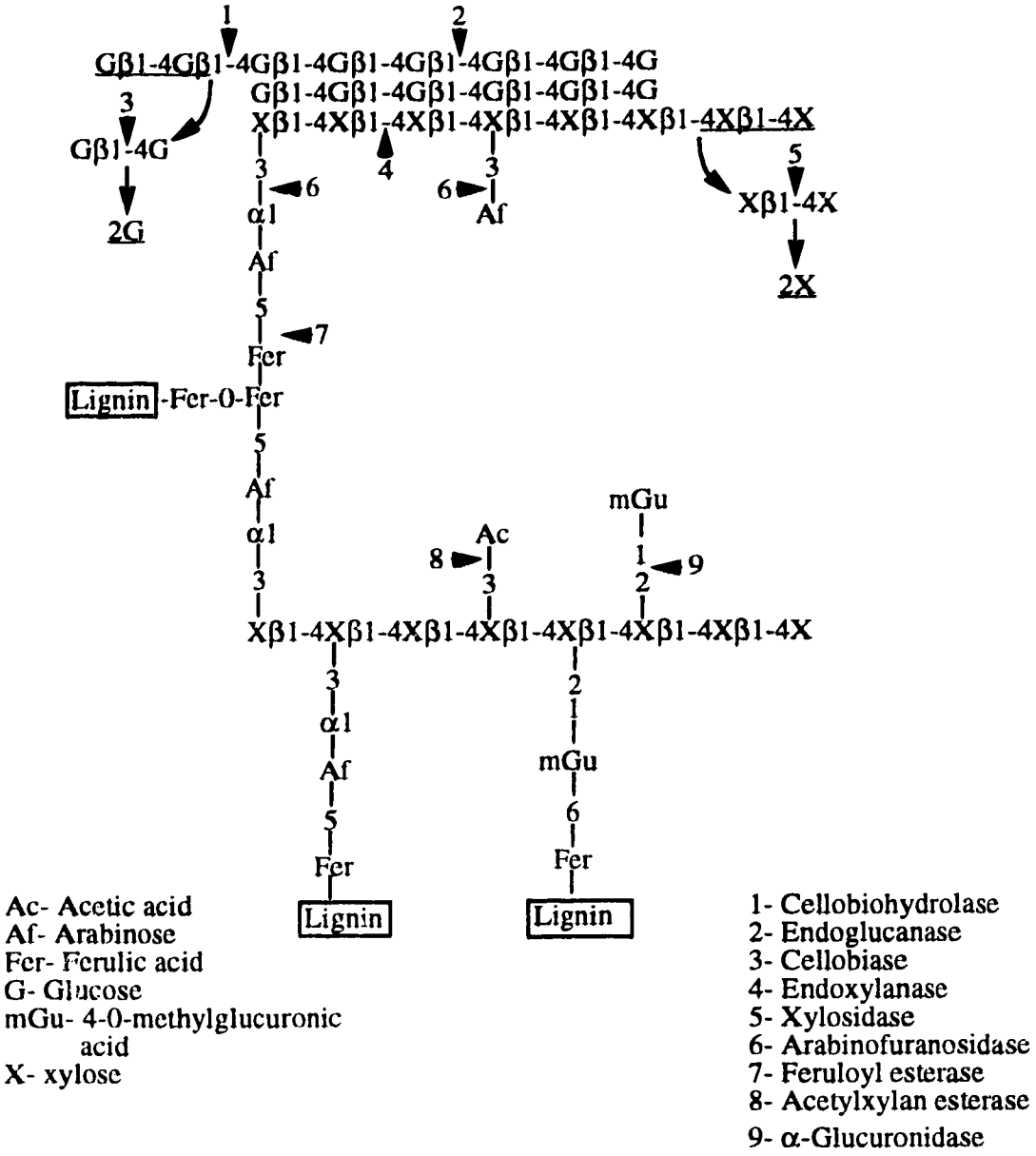

Figure 2.1. Schematic representation of the structure of hemicellulose and cellulose and the major chemical bonds in cell walls. The enzyme cleavage sites and enzymes which cleave these bonds are listed. Adapted from Forsberg *et al.* (1993).

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3.0 Cloning and expression of an amylase gene from *Streptococcus bovis* in *Escherichia coli**

3.1 INTRODUCTION

Streptococcus bovis is one of a group of organisms capable of degrading starch in the rumen (Chesson and Forsberg, 1988; Cotta, 1988). *S. bovis* strains contain both intracellular and extracellular alpha-amylases (Hobson and Macpherson, 1952; Walker, 1965). Expression of amylase activity in *S. bovis* is regulated because inclusion of starch or maltose as the sole energy source increases amylase activity in comparison with activity in medium containing glucose (Cotta, 1988). Cell counts of *S. bovis* in the rumen average 10^7 ml^{-1}, and in animals well adapted to a grain ration the numbers of *S. bovis* do not deviate significantly from numbers in animals fed hay rations (Hungate, 1966). When animals are switched from hay diets to concentrate diets, numbers of *S. bovis* increase substantially, which often results in conditions such as lactic acidosis and bloat (Cheng et al., 1976).

S. bovis plays an important role in starch degradation during periods of high starch concentration in the rumen; thus, it would be beneficial to gain a better understanding of this process. Isolating the amylase genes from *S. bovis* and cloning these genes into *Escherichia coli* will eventually facilitate the study of their regulation in response to changes in starch concentration and other stimuli in the rumen environment (i.e., catabolite repression, feedback). In this report we describe the cloning of an amylase gene from *Streptococcus bovis* 033 and its expression in *E. coli.*

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, plasmids, and growth conditions.

The bacteria used included *Streptococcus bovis* 033 (Rowett Research Institute, Aberdeen, Scotland), *S. bovis* 077 (ATCC 9809), *Butyrivibrio fibrisolvens* 194 and 195 (Orpin et al., 1985), and *Escherichia coli* HB101 and ED8654 (*E. coli* Genetic Stock Center, Department of Biology 255 OML, Yale University). *E. coli* MT614 (Yarosh et al., 1989) was used in the transposon mutagenesis experiments. The vectors used in this study included λgtWESλB (Leder et al., 1977) and pBR322 (Bolivar et al., 1977). The lambda clone λSBA105 and the subclones pSBA01, and pSBA03 were from this study. The anaerobic media and growth conditions for the rumen bacteria in this study were described previously (Stewart and Bryant, 1988). *E. coli* HB101 was grown at 37°C in LB medium and *E. coli* ED8654 was grown at 37°C in TN medium (10 g Bacto-tryptone, 5 g NaCl per liter containing 0.2% maltose and 10 mM $MgCl_2$, pH 7.5). Antibiotics were used at the following concentrations: ampicillin, 100 μg and 50 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; kanamycin, 25 μg and 50 μg ml^{-1}.

3.2.2 Recombinant DNA techniques.

Plasmid preparations, restriction digests, and DNA ligations were done according to standard procedures (Sambrook et al., 1989). Phage DNA was prepared by a method described previously (Hu and Wilson, 1988). *S. bovis* 033 and 077, *B. fibrisolvens* 194 and 195 genomic DNA was prepared as described previously (Hu et al., 1991). Isolation of DNA fragments was done using DEAE membrane

(Schleicher and Schuell, Keene, NH, USA). Subclones were constructed in pBR322 and were mapped using single and multiple restriction digests.

3.2.3 Construction of S. bovis genomic library.

Genomic DNA of *S. bovis* 033 was partially digested with EcoR1 using the protocol described by Hu and Wilson 1988. Fragments of 4-8 kb in size were ligated with λgtWES λB EcoR1 arms at a molar ratio of 1:2 and incubated overnight at 4°C.

The recombinant DNA was packaged as specified by the manufacturers of the *in vitro* packaging kit (Amersham, Oakville, ON, Canada). *E. coli* ED8654 (A_{600}=0.6-1.0) cells were infected with small volumes (2-5 μl) of the recombinant phage and plated on TN plates.

3.2.4 Screening for amylase genes.

To screen the *S. bovis* genomic library for amylase genes, plaques were overlaid with a 0.1% soluble starch--1% agarose (in 50 mM potassium phosphate buffer, pH 6.5) solution and incubated at 27°C or 39°C. The soluble starch was purchased from Sigma Chemical Company (St. Louis, MO, USA). The plates were stained using a Congo red procedure (Teather and Wood, 1982) and then stained with a 0.13% iodine/0.3% potassium iodide solution (Poulsen and Petersen, 1989). Bacterial subclones were screened in the same way. Amylase positive plaques or colonies were identified by a zone of clearing.

3.2.5 Analysis of cloned gene products.

Amylase activity of culture supernatant, cell-free cell extract

and lambda stock was determined using the dinitrosalicylic acid (DNS) procedure (Miller et al., 1960). One unit of amylase activity was expressed as the amount of enzyme releasing glucose equivalents (1 μmol min^{-1}). Specific activity was expressed in units per mg of protein which was determined using the protocol of Bradford (1976). Cell extracts were prepared from 100 ml of overnight cultures of *E. coli, S. bovis*, and *B. fibrisolvens*. Cells were harvested, washed with 50 mM potassium phosphate buffer (pH 6.5) and resuspended in 5 ml of the same buffer. Cells were broken using a French Pressure cell at 12, 000 lb/in^2. This cell suspension was centrifuged at 48, 000 x g for 20 minutes at 4°C, the supernatant was recovered and used as cell-free cell extract in the assays.

Analysis of amylase activity using native polyacrylamide gel electrophoresis was done according to Hu and Wilson (1988). One change was the use of a 0.1% starch--1% agarose overlay.

3.2.6 Transposon mutagenesis.

E. coli MT614 competent cells were transformed with plasmid DNA containing various amylase clones, according to the procedure of Maniatis *et al.* (1982). Colonies containing Tn*5* inserts were identified using a kanamycin gradient plating technique as outlined by Simon (1984). DNA was made from selected colonies and mapped to identify cells containing Tn*5* in the amylase insert.

3.2.7 Southern hybridization.

Genomic DNA of *S. bovis* 033 and 077, and *Butyvibrio fibrosolvens* 194 and 195 was digested completly with EcoR1 or PstI and electrophoresed on 0.8% agarose gel using TBE buffer (Maniatis

et al., 1982). The DNA was then transferred by capillary action to Zeta Probe Nylon Blotting Membrane (Bio-Rad Laboratories, Richmond, CA, USA) using the alkaline procedure detailed by the manufacturers. A nonradioactive DNA labelling and detection kit (Boehringer Mannheim Biochemicals, Laval, PQ, Canada) was used for labelling and detection. Prehybridization and hybridization solutions were made according to the manufacturer of the kit. Prehybridizations, hybridizations and the washes with 0.1% SSC--0.1% SDS were done at 65°C.

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation and subcloning of lambda clones of Streptococcus bovis expressing amylase activity.

To isolate the amylase gene a genomic library of *Streptococcus bovis* 033 was constructed in λgtWES λB and the plaques were screened for expression of amylase activity. Positive plaques were identified by a zone of clearing around them. The host strain, *Escherichia coli* ED8654, did not produce zones of clearing on plates. Ten positive clones were identified from 10,000 plaques that were screened (i.e., 0.1%) and were labelled as λSBA101-110. The plaques were purified three times and plate lysate stocks were made. DNA was isolated from two of the ten clones. When restricted with EcoR1 it was found that both clones contained inserts of approximately 7.5 kb. The 7.5 kb insert from λSBA105 was subsequently cloned into pBR322 to yield pSBA01.

To localize the amylase gene, plasmid pSBA01 was completely digested with HindIII and PstI and various fragments were ligated to pBR322. The results of subcloning indicated that the amylase gene was located within the two PstI fragments of pSBA01 (Fig. 1). When the 2.1 and 1.0 kb PstI fragments of pSBA03 were cloned into pUC18 in both orientations, there was amylase activity produced regardless of orientation. This indicated that transcription of the amylase gene was initiated by an endogenous promoter.

3.3.2 Expression of amylase activity of lambda clone and subclones.

Amylase activity of the λSBA105 clone and the *E. coli*

subclones was much lower than that of the *S. bovis* strain (Table 3.1). The cell extract of the subclones had higher amylase activity than the concentrated supernatant. Osmotic shock treatment (Hughes et al., 1971) of the *E. coli* subclones revealed that the majority of cell-associated amylase activity was contained in the cytoplasmic fraction and not the periplasmic fraction (data not shown). Activity of other amylase genes cloned in *E. coli* has also been shown to be localized to the cytoplasmic fraction and not the periplasmic or extracellular fraction (Horinouchi et al., 1988).

Cell extracts of *S. bovis* and the *E. coli* subclones were run on a native polyacrylamide gel and the gel was screened for amylase activity. *S. bovis* amylase was expressed in the *E. coli* HB101 subclones but they exhibited bands of clearing in addition to the band corresponding to that present in *S. bovis* 033 extracts (Fig. 3.2). *S. bovis* 033 cell extract and supernatant always exhibited one band of clearing. *E. coli* HB101 (pBR322) exhibited no amylase activity when run an native polyacrylamide gels (data not shown). Other researchers also have found multiple forms of enzymes that have been cloned into *E. coli* (Cohen et al., 1990; Hu and Wilson, 1988). These variant forms of the enzyme may result from translational effects which would cause truncated proteins that retain amylase activity. Additionally, these multiple bands could be the result of differences of folding or assembly in *E. coli*.

3.3.3 Transposon mutagenesis of subclones.

Tn*5* transposon mutagenesis was used to study possible causes of the multiple bands of amylase activity in the subclones. We were

interested in determining if insertions of Tn*5* in the amylase gene would result in truncated proteins that were still amylase positive or result in deletion of amylase activity.

It was found that most Tn*5* inserts within the pSBA03 3.1 kb amylase fragment caused a complete loss of amylase activity. Tn*5* inserts were mapped at 0.3, 0.5, 1.0, 1.4, and 1.9 kb (Fig. 3.1). All inserts, except those at 0.3 kb, caused a loss of amylase activity (Fig. 3.3). Cell extracts of the amylase negative cultures exhibited no bands of amylase activity when run on native polyacrylamide gels while the amylase positive cultures still had three bands of activity (data not shown). Transposon Tn*5* causes termination of transcripts initiated by the operon's promoter at most of the sites it inserts at (Wang and Roth, 1988). Since Tn*5* clones did not exhibit bands of clearing the Tn*5* results seem to indicate a translational effect could be causing the multiple bands of activity in the subclones. Also, the 0.3 kb insert did not terminate transcription of the amylase gene providing additional evidence that an endogenous promoter intitiates transcription of the amylase gene.

3.3.4 Identity of amylase gene.

Southern hybridization analysis comfirmed that the cloned amylase gene originated from *S. bovis* 033. The cloned amylase gene was also compared to three other amylolytic rumen bacteria (*S. bovis* 077, and *B. fibrisolvens* 194 and 195) by Southern hybridization. Under the specified experimental conditions, the amylase fragment hybridized to *S. bovis* 033 but not to the other three amylolytic strains. Thus, *S. bovis* 033 amylase gene differs

from the amylases of these other strains under our experimental conditions.

In this report we described the isolation of an amylase gene from *S. bovis* 033 and its cloning and expression in *E. coli*. Further analysis of the enzyme's properties is required to determine its exact molecular weight and if it is an alpha-amylase. It would be of interest to study the stability of the enzyme and its expression in *E. coli* using expression vectors. It also would be beneficial to sequence the gene so that the properties of the gene (e.g., promoter region) could be determined.

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Table 3.1. Amylase activities of *Streptococcus bovis* 033, lambda phage clone, *Escherichia coli* HB101, and the *E. coli* subclones, pSBA01, and pSBA03

Sample	Specific activity (nmol $min^{-1}mg^{-1}$ protein)
S. bovis	
033 SN[a]	16,920
033 CE[a]	5,410
λSBA105 lysate	110
E. coli	
HB101 SN	0
HB101 CE	25
E. coli	
pSBA01 SN	29
pSBA01 CE	86
pSBA03 SN	38
pSBA03 CE	230

[a]CE=cell extract, SN=supernatant; samples were prepared and amylase activity was measured as outlined in Materials and Methods. Assay was done three times on sets of two of each sample.

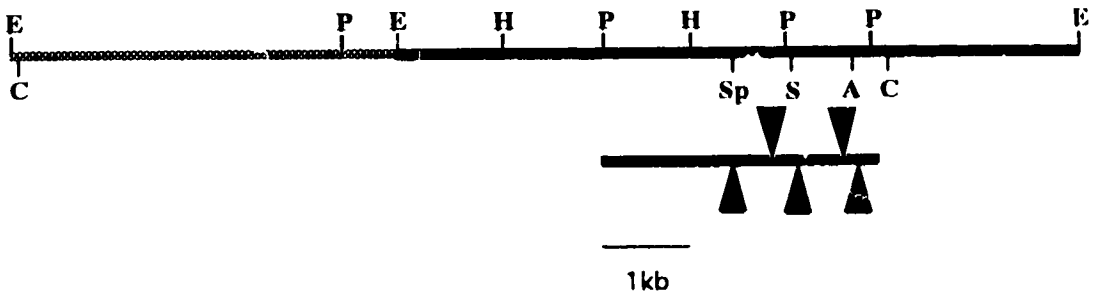

Figure 3.1. Restriction map of subclone pSBA01. The bold line under pSBA01 indicates the approximate region of the amylase gene. This PstI region is contained in subclone pSBA03. Arrowheads indicate positions of Tn*5* inserts. Striped arrows represent amylase positive inserts and black arrows represent amylase negative inserts. A=AvaI; C=ClaI; E=EcoR1; H=HindIII; P=PstI; S=SalI; Sp=SphI. Shaded line represents pBR322 and the bold line represents the amylase insert.

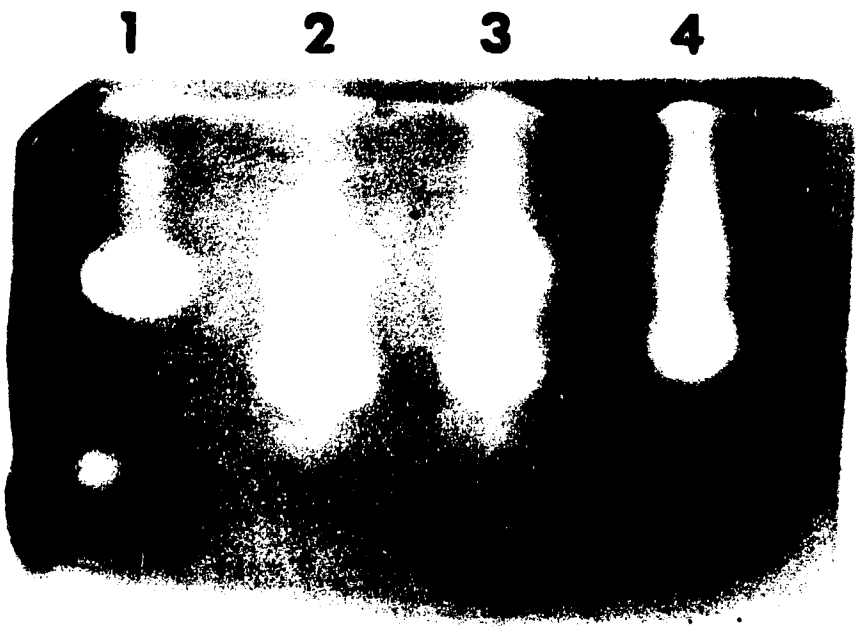

Figure 3.2. Overlay gel from native polyacrylamide gel showing amylase activity of *Streptococcus bovis* 033 and the subclones. Lane 1=*S. bovis* 033 supernatant (1.46 μg), lane 2=pSBA01 cell extract (60 μg), lane 3=pSBA02 cell extract (65 μg), and lane 4=pSBA03 cell extract (26 μg). Amount of protein loaded of each sample is indicated in brackets.

Figure 3.3. Comparison of effects of Tn*5* insertion into the 3.1 kb amylase fragment of subclone pSBA03. (a) pSBA03::Tn*5*E, which has Tn*5* inserted at 0.3 kb, still exhibits amylase activity. (b) pSBA03::Tn*5*, which has Tn*5* inserted into the 3.1 kb insert at 0.5 kb, exhibits no amylase activity (i.e., no zone of clearing).

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4.0 Development of shuttle vectors for *Butyrivibrio fibrisolvens* and their transfer by electroporation

4.1 INTRODUCTION

Molecular biology studies of rumen microorganisms have concentrated on the cloning of polysaccharidases from some of the predominant rumen bacteria and fungi into *Escherichia coli.* These studies have elucidated the number and types of enzymes involved in plant polysaccharide degradation. The lack of vectors and transfer systems for rumen bacteria has meant that the regulation and expression of cloned genes in rumen bacteria cannot be studied. Additionally, in order to develop genetically modified rumen bacteria appropriate vectors and transfer systems are required.

The rumen bacterium *Butyrivibrio fibrisolvens* is metabolically versatile and one of the predominant bacteria isolated from the rumen. Members of this genus are involved in the digestion of starch, hemicellulose, cellulose, lipids, proteins, and pectin (Bryant and Small, 1956). Due to its metabolic diversity *B. fibrisolvens* is present in the rumen under a variety of feeding conditions. The characterisitics of *B. fibrisolvens* suggest that this organism is a suitable candidate for genetic modification.

Enzymes involved in the degradation of cellulose and hemicellulose have been isolated from *B. fibrisolvens* and genes coding for some of these enzymes have been cloned and their expression studied in *Escherichia coli* (Sewell et al., 1989; Hazlewood et al., 1990; Mannarelli et al., 1990; Lin and Thomson, 1991; Hespell and O'Bryan, 1992). The analysis of their regulation,

and the regulation of foreign genes, by *in vitro* mutations and reintroduction into *B. fibrisolvens* requires shuttle vectors and a transformation system specific for this organism.

Transfer systems based on the technique of electroporation have proven useful for bacteria that could not be transformed using conventional transformation procedures (Shigekawa and Dower, 1988). Electroporation involves application of a high-intensity electrical field to bacterial cells resuspended in a low conductivity buffer which induces membrane breakdown in the cells (Chang, 1992). The cell membranes become highly permeable to exogenous DNA in solution with the cells and after a recovery period in growth medium a percentage of the cell population will contain plasmid DNA. Electroporation was the first technique used to transform the Gram negative rumen bacterium *Prevotella (Bacteroides) ruminicola* (Thomson and Flint, 1989). Recently electroporation was used to introduce plasmids into *B. fibrisolvens* strains H17c and AR10 (Ware, et al., 1992; Whitehead, 1992). Although the transformation frequencies were low these studies demonstrated that it is possible to transform rumen bacteria using electroporation.

The focus of this work was to develop plasmid vectors for *B. fibrisolvens* based on an endogenous *B. fibrisolvens* 194 plasmid, pBF194. Vectors were developed that, in addition to pBF194, contained well characterized plasmids from Gram positive or Gram negative bacteria. The use of electroporation as a method to transform selected *B. fibrisolvens* strains, and modifications to the procedure, are described. The introduction and expression of the *S.*

bovis 033 amylase and a chloramphenicol resistance gene, using two of the constructed shuttle vectors, is also described.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used are listed in Tables 4.1 and 4.2. *Escherichia coli* strains were cultured on LB medium (10g tryptone, 5g yeast extract, 10g NaCl per liter) at 30°C. *Butyrivibrio fibrisolvens* strains were cultured on: (i) RGM medium (Hespell et al., 1988) with 0.2% glucose (RGMG) or 0.2% starch (RGMS); (ii) Dehority's medium (Scott and Dehority, 1965) containing 0.2-1.0% glucose; (iii) or M2 medium (Hobson, 1969). Media were prepared anaerobically according to the original technique described by Hungate (1950) and later modified by Bryant (1972). Cultures were grown in an anaerobic glovebox (Forma Scientific, Marietta, OH) with an atmosphere of 85% nitrogen, 10% carbon dioxide, and 5% hydrogen. *Enterococcus faecalis* strains were cultured aerobically, without shaking, at 37°C on M17 medium (BDH Inc., Darmstadt, FRG). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 1-10 μg/ml for pLRS05, pC194, pLRS07Km/Cm, and pRC vectors and 100 μg/ml for pSA3; erythromycin, 100 μg/ml for pHP13, 200 μg/ml for pSA3, and 5-10 μg/ml in *B. fibrisolvens* electroporation experiments with pLRS05 and pLRS06; kanamycin, 20-30 μg/ml; tetracycline, 10 μg/ml.

4.2.2 DNA preparation, analysis, and manipulation

Plasmid DNA was isolated from *E. coli, B. fibrisolvens* and *Bacillus subtilis* using a modified alkaline lysis procedure (Sambrook, et al., 1989). In the case of *B. fibrisolvens* and *B.*

subtilis, once cells were resuspended in lysozyme solution they were incubated at 37°C for 15 minutes or 30 minutes, respectively. Genomic DNA was isolated as described previously (Hu et al., 1991). Single-stranded DNA of pUBLRS was made according to the procedure in Sambrook *et al.* (1989). The host strain used was *E. coli* XL1-Blue and the helper phage used for single strand induction was M13K07.

Restriction endonuclease digests were done according to procedures specified by the manufacturer (Gibco/BRL, Gaithersburg, MD). DNA preparations and digests were analyzed by electrophoresis on agarose gels (0.8% agarose in Tris-borate buffer [TBE; Sambrook et al., 1989]) run in TBE. DNA fragments were detected by staining gels in distilled water containing ethidium bromide (1 μg/ml) and visualized by UV transillumination. Isolation of DNA fragments from agarose was done using Prep-A-Gene (Bio-Rad Laboratories, Richmond, CA). Ligations with T4 DNA ligase (Gibco/BRL) were done according to standard procedures (Sambrook et al., 1989) while blunt end ligations were done according to the protocol of Cobianchi and Wilson (1987).

The presence of plasmid DNA in *E. coli* and *B. fibrisolvens* transformants could be screened initially using a modified Eckhardt technique (Hynes et al., 1985). For *E. coli* transformants, Eckhardt gels could be run using cells from plates. Cells were scraped off plates on the tip of sterile pipet tips (10-200 μl size) and the cells resuspended in E1-lysozyme solution (10% sucrose, 10 μg/ml RNase in TBE, and 100 μg/ml of lysozyme [added fresh]). This suspension is loaded directly onto 0.8% agarose-1% SDS gels, in TBE.

Southern hybridization analysis was used to verify the presence of vectors in transformants. Genomic DNA of selected transformants was digested with the appropriate restriction endonuclease and resolved by electrophoresis. The DNA was transferred to Zeta Probe® blotting membrane (Bio-Rad Laboratories) using the alkaline transfer procedure outlined by the manufacturer. A nonradioactive DNA labelling and detection kit (Boehringer Mannheim Biochemicals, Laval, PQ) was used for labelling of probes and color detection.

4.2.3 Transformation procedures

Plasmid DNA and ligation products were transformed into $CaCl_2$ competent *E. coli* cells (Sambrook et al., 1989) or transformed into electrocompetent *E. coli* using the electroporation procedure outlined by the manufacturer of the Gene Pulser (Bio-Rad Laboratories). Transformations were plated onto selective LB medium. The general electroporation protocol for *B. fibrisolvens* is as outlined by Whitehead (1992). At least 1-2 μg of prepared plasmid DNA was used in each cell preparation for electroporation. Modifications to the protocol were made during the course of this project to determine if this would result in transformation of *B. fibrisolvens* strains 193, 194, and 195. These modifications are as follows: (i) Strains were grown in M2, DG, or RGMG medium to determine whether different media had an effect on transformation. (ii) Wash buffers used included 10% glycerol, 10% glycerol-1mM $MgCl_2$, and 10% polyethylene glycol (PEG; MW 8000). (iii) Prepared electrocompetent cells of the *B. fibrisolvens* strains were heat

treated at 48°C or 55°C for 15 minutes to inactivate host restriction endonucleases. (iv) Electroporation parameters were varied, including field strengths of 6.25, 10, and 12.5 kV/cm and capacitor resistances of 200-1000 ohms (Ω). (v) Plasmid DNA was isolated from *E.coli* hosts HB101, DH5α,and GM272 which vary in their restriction-modification genotypes. (vi) Single-stranded DNA of pUBLRS was isolated and used in electroporation experiments with strains 194 and H17c.

Electroporation of *E. faecalis* was done according to the protocol of Cruz-Rodz and Gilmore (1990).

4.2.4 Screening of amylase and xylanase activity

Bacterial colonies containing pJHA and pUBLRSA were screened for amylase activity according to the protocol in Clark *et al.* (1992). Screening of xylanase activity of pUBCX.1 transformants was done according to the procedure outlined in Hu *et al.* (1991). Cell extract and supernatant from H17c (pUBLRSA) transformants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a Mini-PROTEAN II electrophoresis system (Bio-Rad Laboratories). Crude cell extract was prepared from 25 ml of overnight cultures. Cells were centrifuged at 12, 000 x g for 15 minutes at 4°C and washed once with 50 mM sodium phosphate buffer containing 5mM $CaCl_2$ (pH 6.5). Cells were resuspended in 1 ml of the same buffer and cells were disrupted by sonication on ice (30 s bursts for 2 minutes) using an Artek Systems Sonic 300 dismembrator sonicator (Farmingdale, NY, USA) with an intermediate tip at 60% intensity. This cell suspension was centrifuged for 30 minutes at 12, 000 x g

at 4°C. Gels were run at 150V for 1-2 hours at room temperature. Activity was detected *in situ* by including soluble starch (0.1%) in the gels. After electrophoresis the segments of the gels containing the molecular weight standards were cut off and stained in Coomassie blue G-250. The rest of the gels were renatured by washing for 1 hour in three changes of 1% TRITON-X100 and then incubated in 0.05M sodium phosphate-5mM $CaCl_2$ buffer (pH 6.5) for 1 hour at 4°C. Gels were incubated in fresh buffer at 37°C for 12-16 hours and then stained with a 0.13% iodine/0.3% potassium iodide solution to detect amylase activity.

4.3 RESULTS

4.3.1 Construction of shuttle vectors

Shuttle vectors, pLRS03, pLRS05, pLRS06, and pRC, were developed for *Butyrivibrio fibrisolvens* based on an endogenous *B. fibrisolvens* plasmid and other well characterized Gram positive and Gram negative plasmid vectors (Figs. 4.1-4.4). Initially, selected *B. fibrisolvens* strains were screened for plasmids using a modified Eckhardt technique (Hynes, et al., 1985). A small plasmid of approximately 3.0 kb in size, designated pBF194, was identified in *B. fibrisolvens* strain 194 and was subsequently isolated and characterized by restriction mapping (Fig. 4.1). Four unique sites were located on pBF194 and two of these, Cla1 and Xba1, were used for cloning pBF194 into other plasmid vectors. The BamH1 site of pBF194 was only identified when pBF194 was contained in a shuttle vector that could be propagated in *E. coli*. pBF194 isolated directly from *B. fibrisolvens* 194 was resistant to digestion with BamH1 as was 194 genomic DNA, indicating that there may be a BamH1 methylase present in strain 194.

All of the vectors were introduced into strains of *E. coli* using $CaCl_2$ transformation or electroporation. These shuttle vectors incorporated a variety of replicons and numerous antibiotic resistance determinants that could potentially be expressed in *B. fibrisolvens* (Fig. 4.2-4.4). The pRC shuttle vectors consist of the shuttle vector pLRS03 and the promoter-probe vector pKK232-8 containing various sizes of *B. fibrisolvens* promoter fragments. The first step in this construction was to isolate potential promoter

fragments from a cloned *B. fibrisolvens* 194 xylanase gene. The xylanase gene was digested with HindIII and these fragments were cloned into the multiple cloning site, which is 5'-proximal to the promoterless CAT gene, of pKK232-8. Three chloramphenicol resistant clones, pKKBFX67, 68 and 74, with different sizes of promoter fragments were identified. pLRS03, containing pBR322 and pBF194, was cloned into each of these promoter clones to yield the pRC vectors. The purpose of these constructs was to provide vectors that contained a *B. fibrisolvens* replicon and an antibiotic resistance gene driven by putative *B. fibrisolvens* promoters.

Whitehead (1992) was the first to report transformation of *B. fibrisolvens* strain H17c, using electroporation, with the *Bacillus subtilis* shuttle vector pBS42, which contains the *Staphylococcus aureus* plasmid pUB110. We were able to transform strain H17c with pUB110 and demonstrate the presence of this plasmid in H17c (Table 4.3). Based on these results, shuttle vectors based on pUB110 were constructed which could replicate in *E. coli* (Clark et al., 1994). These vectors have the advantage over the previous vectors, of containing an antibiotic resistance gene (neomycin/kanamycin) that is known to be expressed in *B. fibrisolvens.* In addition to an *E. coli* replicon (pBluescriptII), the shuttle vector pUBLRS contains pBF194 and the pUB110 origin of replication, which is functional in strain H17c, while pLRS07Km only contains pBF194 and the neomycin/kanamycin resistance gene of pUB110.

4.3.2 Electroporation as a method to introduce shuttle vectors into strains of *B. fibrisolvens*

Initial electroporation experiments were done using *B. fibrisolvens* strains 193, 194 and 195 and shuttle vectors pLRS03, pLRS05, pLRS06, pRC106, and pRC231. The first electroporation protocols were similar to those used in *E. coli.* None of the shuttle vectors could be introduced into strains 193, 194, and 195 (Table 4.3). Strains 194 and H17c were used in electroporation experiments attempting to introduce vectors pUBLRS and pLRS07Km into *B. fibrisolvens.* These two vectors could only be introduced into strain H17c (Table 4.3).

Various modifications to the electroporation protocol, as outlined in Materials and Methods, did not result in transformation of *B. fibrisolvens* strains 193, 194, or 195. *B. fibrisolvens* H17c was the only strain which could be transformed with plasmid DNA (Table 4.3). Using pUBLRS isolated from *E. coli* GM272, optimal electroporation parameters for strain H17c were determined to be 2.0 kV, 25 μF, and 200Ω (Table 4.4).

4.3.3 Introduction of foreign genes into shuttle vectors

Using electroporation the plasmid pUB110 and vectors based on it, pLRS07Km and pUBLRS, could be introduced into *B. fibrisolvens* H17c (Clark et al., 1994). To determine whether these plasmids could be utilized as shuttle vectors, foreign genes were cloned into them. pBCX600, a vector containing a xylanase gene from *Bacillus circulans* (Yang et al., 1989), was cloned into pUB110 to give the vector pUBCX.1 (Fig. 4.5). Two vectors, pUBLRSA and pJHA (Fig. 4.6),

contain a *Streptococcus bovis* amylase gene (Clark, et al., 1992). pLRS07Km contains the chloramphenicol gene of the *S. aureus* plasmid pC194 (Fig. 4.7). These vectors were used in electroporation experiments to determine if they could be used to introduce foreign genes into *B. fibrisolvens.*

pUBCX.1 and pJHA transformants were not detected even after numerous attempts to introduce them into *B. fibrisolvens* H17c. pUBCX.1 and pJHA could be introduced into *Enterococcus faecalis* JH2-2E using electroporation and expression of amylase and xylanase activity was evident when transformants were screened (data not shown).

pLRS07Km/Cm and pUBLRSA transformants of *B. fibrisolvens* H17c were detected at a very low frequency ($<10^1$ CFU/μg of DNA). In order to detect these transformants, the recovery mixture of electroporated cells was subcultured in 5ml of RGMG medium containing 30 μg/ml of kanamycin and subsequently plated on RGMG plates containing 5 μg/ml of chloramphenicol and 20 μg/ml of kanamycin for pLRS07Km/Cm or just kanamycin for pUBLRSA. pLRS07Km/Cm and pUBLRSA could be detected in selected kanamycin resistant colonies screened on Eckhardt gels. pLRS07Km/Cm DNA isolated from H17c transformants was digested with EcoR1 and Sal1 and compared to pLRS07Km/Cm DNA isolated from *E. coli* GM272. The digest patterns were identical indicating that there had been no rearrangement of the plasmid in H17c. Southern hybridization analysis of pUBLRSA transformants of H17c, using the cloned *S. bovis* amylase gene as a probe, verified the presence of the *S. bovis*

amylase gene in these clones (data not shown). pLRS07Km and pUBLRS can therefore be used as shuttle vectors to introduce foreign genes into H17c and possibly other strains of *B. fibrisolvens* that are amenable to electroporation. In addition, since pUBLRS is a mobilizable vector (Clark et al., 1994), strains unable to be transformed by electroporation can be transformed via conjugation.

4.3.4 Expression of foreign genes in *B. fibrisolvens* H17c

H17c transformants containing pLRS07Km/Cm expressed resistance to 5-10 μg/ml of chloramphenicol, while wild type H17c was sensitive to these levels of antibiotic. The presence of the *S. bovis* amylase gene in H17c (pUBLRSA) transformants was verified by Southern hybridization analysis and it was of interest to determine if the gene was being expressed in H17c. Zymogram analysis of cell extracts prepared from H17c (pUBLRSA) recombinants and wild-type H17c revealed additional bands of amylase activity in the recombinants (Fig. 4.8). A difference in expression of amylase activity was detected between cells grown in starch medium and cells grown in glucose medium (Fig. 4.8). These preliminary results seem to indicate that the *S. bovis* amylase gene is being expressed in *B. fibrisolvens* H17c.

4.4 DISCUSSION

Transformation of *Butyrivibrio fibrisolvens* is limited to certain strains as was shown in this project. Of the four *B. fibrisolvens* strains tested only strain H17c was amenable to transformation by electroporation indicating that there is strain to strain variability and that there is not one general protocol that will be useful for all strains of *B. fibrisolvens.* It has been observed that Gram negative bacteria are generally easier to transform using electrical methods than are Gram positive bacteria (Trevors et al., 1992). With optimal electroporation conditions for *Escherichia coli,* transformation efficiencies of 10^{10} transformants/μg of DNA can be obtained (Dower et al., 1992). This is in contrast to efficiencies of 10^{6} transformants/μg of DNA in Gram positive bacteria, which is considered very high in most cases (Trevors et al., 1992). Often only a few colonies are recovered and generally only 1% of the population is ever transformed (Dower et al., 1992). Microscopic examination of the cell wall structure of *B. fibrisolvens* strains has revealed the lack of an outer membrane and the presence of a thin, Gram positive-like, cell wall structure (Cheng and Costerton, 1977). Since *B. fibrisolvens* appears to be a Gram positive bacterium rather than Gram negative as it was originally considered, the lack of success in transformation by electroporation may not be that unusual.

There can be many barriers to successful electroporation of an organism and in the situation where there is no established protocol it can be difficult to make appropriate modifications to overcome these barriers. Conditions such as growth media, wash buffers,

electroporation parameters, and the source of DNA need to be examined. Growth medium may have an effect on cell wall density that could hinder transformation, so in these experiments strains were grown on three different types of media. DG and M2 are rich growth media containing rumen fluid while RGMG, also a complex medium, does not. Including agents in the media that affect cell wall structure, such as threonine and glycine, has been known to increase transformation frequencies in Gram positive bacteria (Chassy et al., 1988; Holo and Nes, 1989) and the usefulness of glycine in some of the electroporation experiments was examined. The use of different media and adding glycine to growing cultures had no effect on electroporation of strains 193, 194, and 195.

A comparison of wash buffers was done and in the case of H17c the use of 10% polyethylene glycol (PEG) buffer increased the frequency of transformation, by pUB110, 10-fold over 10% glycerol. This phenomenon has also been observed for both *Clostridium perfringens* and *C. acetobutylicum* where transformation efficiency was increased up to 100-fold in the presence of 10% PEG (Dower et al., 1992). Other modifications included using a variety of field strengths and resistances, as field strength and the time constant of the electrical pulse are considered to be the most important electrical variables affecting electroporation (Shigekawa and Dower, 1988). These modifications did not result in transformation of *B. fibrisolvens* strains 193, 194, or 195. For strain H17c optimal electroporation parameters were determined, using 0.2 cm cuvettes, to be 200Ω resistance, 2.0 kV, and 25 μF. These parameters

corresponded to an average time constant of 4.1 msec and a field strength of 10 kV/cm.

The presence of host restriction systems in *B. fibrisolvens* could interfere with transfer of plasmid DNA, a problem which cannot be directly circumvented by the technique of electroporation. Restriction-modification systems have been identified in the rumen bacteria *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* FD-1 (Lee et al., 1992; Morrison et al., 1992). Although restriction-modification systems haven't been identified in *B. fibrisolvens* we have noted that plasmid DNA and genomic DNA isolated from *B. fibrisolvens* strain 194 is resistant to digestion with BamHI, indicating the presence of a BamHI modification system in this strain. Another obstacle to electroporation can be the secretion of non-specific nucleases into the electroporation medium (Shigekawa and Dower, 1988). Selected strains of rumen bacteria have been shown to possess high nuclease activity which could degrade introduced DNA (Flint and Thomson, 1990).

DNA isolated from dam-, dcm- mutants of *E. coli,* rather than wild type strains, often permits transformation (Mazodier and Davies, 1991). The vectors used in electroporation experiments were isolated from a variety of *E. coli* hosts with various modification-restriction genotypes. DNA isolated from *E. coli* GM272, a restriction-modification deficient strain, was introduced into strain H17c but not into any of the other strains tested. This result indicated that the origin of the plasmid DNA can have an effect on the ability to introduce DNA into *B. fibrisolvens.* Heat

treatment of *B. fibrisolvens* recipients, which should inactivate host restriction endonucleases, did not result in transformants. Another modification was to use single-stranded DNA (ssDNA) which is resistant to restriction endonucleases (Sambrook et al., 1989). pUBLRS transformants of H17c were obtained using dspUBLRS but no transformants were detected using sspUBLRS.

Developing vectors for an organism, for which no vectors or transfer systems exist, requires that they have functional replication origins that will be recognized by the host and antibiotic resistance genes that will be expressed. The vectors developed in this project contained a variety of replication origins and antibiotic resistance genes. Additionally, pBF194 was cloned into the various vectors using two unique restriction sites which would potentially avoid the possibility of having interrupted the replication origin of this plasmid. Of the vectors developed only those based on pUB110 and pBF194 were introduced into strain H17c by electroporation indicating that vectors for *B. fibrisolvens* should be based on Gram positive replicons and antibiotic resistance genes. The vectors pUBLRS, a mobilizable shuttle vector, and pLRS07Km will prove useful for *B. fibrisolvens* H17c and possibly other strains of *B. fibrisolvens.*

Table 4.1. Bacterial strains used.

Strains	Relevant characteristics	Reference
E. coli		
DH5α	F- ϕ80d*lac*ZΔM15 *end*A1 *rec*A1 *thi*-1 *hsd*R17(r_k- m_k+) *sup*E44 λ- *gyr*A96 *rel*A1 Δ(*lac*ZYA-*arg*F)U169	BRL
DH10B	F- *mcr*A Δ(*mrr hsd*RMS *mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara, leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L	BRL
GM272	F- *hsd*S21 *dam*-3 *dcm*-6 *met*B1 *gal*K2 *gal*T22 *lac*Y1 *tsx*-78 *mtl*-1 *sup*E44 *thi*-1 *ton*A31	Marinus *et al.* (1983)
HB101	F- *mcr*B *mrr hsd*S20(r_B-m_B-) *rec*A13 *leu*B6 *ara*-14 *pro*A2	BRL
XL1-Blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F *pro*AB *lac*Iq ZΔM15 Tn*10* (tetr)]	BRL
B. fibrisolvens		
H17c	nalr smr	Hespell and Whitehead (1991)
193	nalr	Orpin *et al.* (1985)
194	nalr; pBF194	Orpin *et al.* (1985)
195	nalr	Orpin *et al.* (1985)
E. faecalis		
JH2-2	rifr fusr	Hespell and Whitehead (1991)
JH2-2E	rifr fusr emr; emr mutant of JH2-2	This study
B. subtilis		
BD366	kanr nmr; pUB110; *thr*-5 *trp*C2	Bacillus Genetic Stock Center

Abbreviations: em=erythromycin; fus=fusidic acid; kan=kanamycin; nal=naladixic acid; nm=neomycin; rif=rifampicin; sm=streptomycin; tet=tetracycline

Table 4.2. Plasmids used

Plasmids	Relevant characteristics	Source or reference
pBluescriptIIKS/SK+	2.96 kb; amp^r	Stratagene
pBR322	4.4 kb; amp^r tet^r	Bolivar *et al.* (1977)
pKK232-8	5.1 kb; amp^r promoterless CAT gene	Brosius (1984)
pHP13	4.9 kb; cm^r em^r	Haima *et al.* (1987)
pSA3	10.2 kb; cm^r em^r tet^r	Dao and Ferretti (1985)
pC194	2.9 kb; cm^r	Horinouchi and Weisblum (1982)
pAM120	21.4 kb; amp^r tet^r; Tn916	Jones *et al.* (1987)
pBCX600	4.0 kb; amp^r; xylanase gene of *Bacillus circulans*	Yang *et al.* (1989)
pBF194	3.0 kb; cryptic *B. fibrisolvens* plasmid	This study
pCBS(B4)	4.6 kb; amp^r cm^r; 1.6 kb ClaI Cm^r fragment of pC194 cloned in pBluescriptII SK+	This study
pLRS03	7.4 kb; amp^r tet^r; pBR322-pBF194 construct	This study
pLRS05	8.55 kb; cm^r em^r; pBF194-pHP13 construct	This study
pLRS06	13.2 kb; cm^r em^r tet^r; pSA3-pBF194 construct	This study
pRC106/231/339	12.75/12.95/12.70 kb amp^r cm^r; contain pLRS03 and pKK232-8 with promoter fragments of *B. fibrisolvens* xylanase (from pBFX01)	This study
pBFX01	*B. fibrisolvens* 194 xylanase (6.2 kb) in pBR322	Hu and Cheng, (unpublished)
pSBA03	7.5 kb; amp^r tet^r; 3.1 kb *S. bovis* amylase in pBR322	Clark *et al.* (1992)
pBSA6	6.1 kb; amp^r; PstI amylase fragment of pSBA03 in pBSIISK+	This study
pUBCX.1	8.6 kb; amp^r nm^r/km^r pm^r; pBCX600-pUB110 construct	This study
pJHA	10.6 kb; amp^r nm^r/km^r; pUB110 cloned into pBSA6	This study
pUBLRSA	14.2 kb; amp^r nm^r/km^r; contains 3.6 kb PvuII amylase fragment of pBSA6 in pUBLRS[a]	This study
pLRS07Km/Cm	10.0 kb; amp^r cm^r nm^r/km^r; chloramphenicol gene of pCBS(B4) cloned into pLRS07Km[a]	This study

Abbreviations: amp=ampicillin; cm=chloramphenicol; em=erythromycin; nm/km= neomycin/kanamycin; pm=phleomycin; tet=tetracyline; CAT=chloramphenicol acetyltransferase
(a) These vectors described in chapter 5 and Clark et al., 1994.

Table 4.3. Vectors used in electroporation experiments with *B. fibrisolvens* strains 193, 194, 195, and H17c [a]. Transformation frequencies for H17c are expressed in CFU/μg of DNA

Plasmids	Strains 193	194	195	H17c
pLRS03	0	0	0	ND[b]
pLRS05	0	0	ND	0
pLRS06	ND	0	0	ND
pRC106	0	ND	ND	ND
pRC213	0	0	0	ND
pAM120	0	0	ND	0
pBS42	ND	0	ND	10[c]
pUB110	ND	0	ND	2.60×10^1
pUBLRS	ND	0	ND	3.28×10^2
sspUBLRS[d]	ND	0	ND	0
pLRS07Km	ND	0	ND	5.00×10^1

(a) experiments done 2-5 times
(b) experiments not done with this combination of plasmid and strain
(c) from Whitehead (1992); expressed as transporants/experiment
(d) single-stranded DNA of pUBLRS made according to protocol in Materials and Methods

Table 4.4. Effect of various voltage and resistance settings on cell survival, time constants, and transformation frequency of *B. fibrisolvens* H17c using the shuttle vector pUBLRS[a].

Voltage (kV)	Resistance (Ω)	Field strength (kV/cm)[b]	Time constant (msec)	Cell survival (CFU/ml)[c]	Frequency (CFU/μg)[d]
2.5	100	12.5	1.8	3.08×10^8	80
2.5	200	12.5	4.5	2.29×10^8	--[e]
2.5	400	12.5	8.1	2.60×10^7	20
2.5	600	12.5	10.5	3.77×10^6	0
2.5	800	12.5	14.3	2.94×10^7	113
2.0	100	10.0	2.4	2.20×10^8	100
2.0	200	10.0	4.5	2.00×10^8	207
2.0	400	10.0	8.6	1.80×10^7	13
2.0	600	10.0	11.5	6.00×10^7	60
2.0	800	10.0	13.8	3.84×10^7	7

(a) pUBLPS was isolated from *E. coli* GM272.
(b) 0.2 cm electrode gap cuvettes (Bio-Rad) were used.
(c) The starting cell count was 3.40×10^9.
(d) The approximate concentration of pUBLRS used for each sample was 1.5 μg.
(e) There were contaminants on this plate so no value could be determined for transformation frequency.

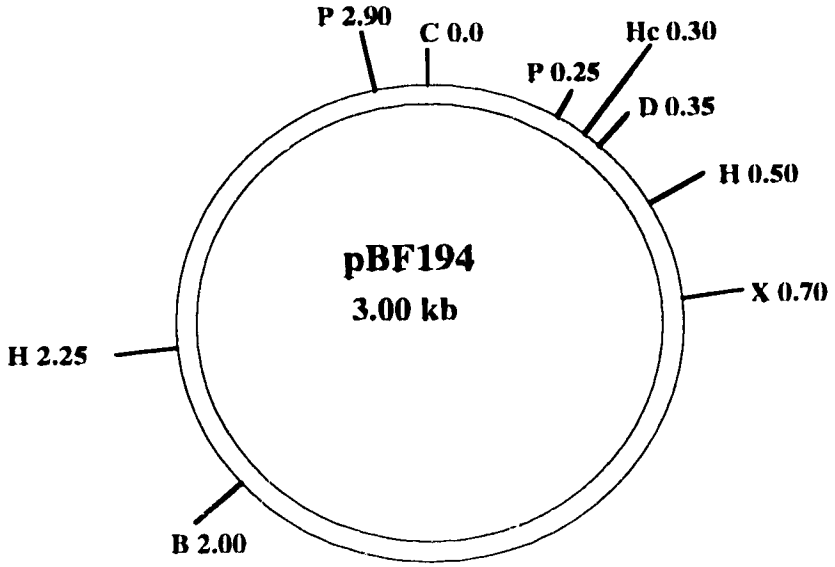

Figure 4.1. Restriction map of the *B. fibrisolvens* 194 plasmid pBF194. Enzymes that cleaved the plasmid are indicated on the map. pBF194 was not digested by the following enzymes: AccI, ApaI, AvaI, BclI, EcoRI, EcoRV, KpnI, SalI, SmaI, SphI, SstI, SstII, and XhoI. pBF194 is not digested by BamHI when the DNA is isolated from *B. fibrisolvens* but will cut the plasmid when propagated in *E. coli*.
B=BamHI; C=ClaI; D=DraI; H=HindIII; Hc=HincII; P=PstI; X=XbaI.

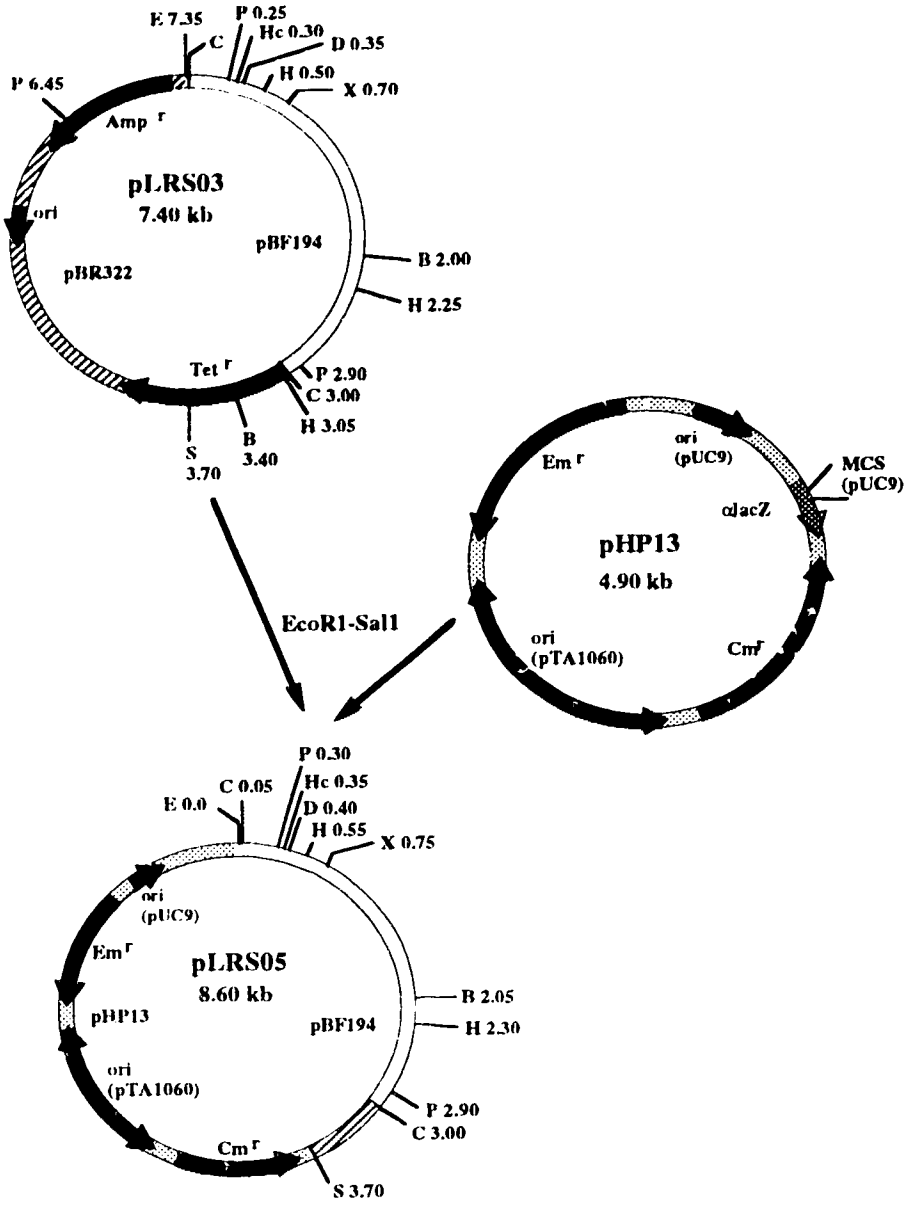

Figure 4.2. Plasmid pLRS03 contains pBF194 cloned into the ClaI site of the *Escherichia coli* vector pBR322. The 3.5 kb EcoRI-SalI fragment of pLRS03, containing pBF194, was cloned into the *Bacillus subtilis* vector pHP13 to yield pLRS05. The erythromycin (Em) and chloramphenicol (Cm) resistance genes of pHP13 originate from the *Staphylococcus aureus* plasmids pE194 and pC194, respectively. B=BamHI; C=ClaI; E=EcoRI; H=HindIII; P=PstI; S=SalI; X=XbaI; ori=origin of replication; amp=ampicillin; lacZ=lac promoter for ß-glucosidase transcription.

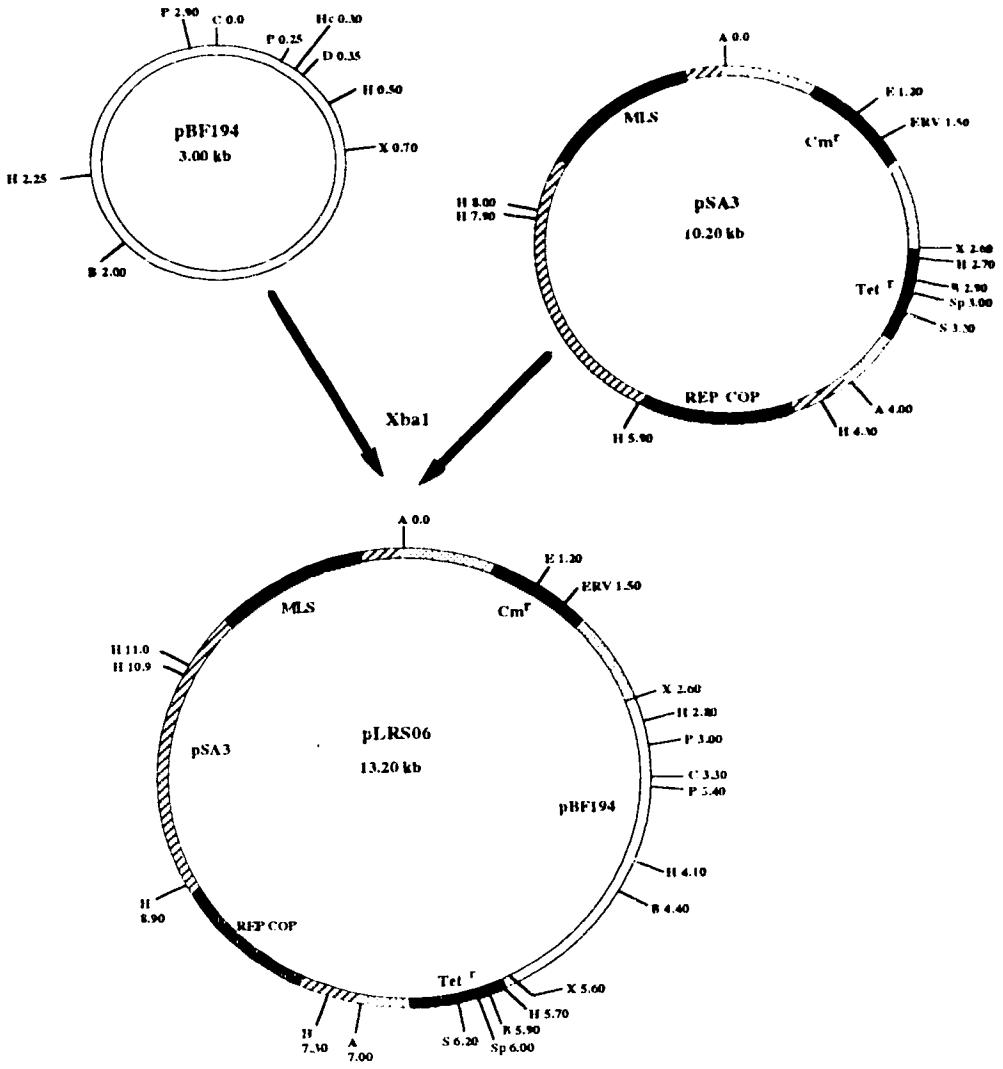

Figure 4.3. Construction of pLRS06. pBF194 was cloned into the unique XbaI site of the *Streptococcus-Escherichia coli* shuttle vector pSA3. The chloramphenicol (Cm) and tetracycline (Tet) resistance genes of pSA3 originate from the *E. coli* plasmid pACYC184 and the MLS (macrolide linocosamide streptogramin B resistance) determinant, which encodes for erythromycin resistance, originates from the streptococcal plasmid pGB305. A=AvaI; B=BamHI; C=ClaI; D=DraI; E=EcoRI; ERV=EcoRV; H=HindIII; Hc=HincII; P=PstI; S=SalI; Sp=SphI; X=XbaI; REP/COP=replication origin.

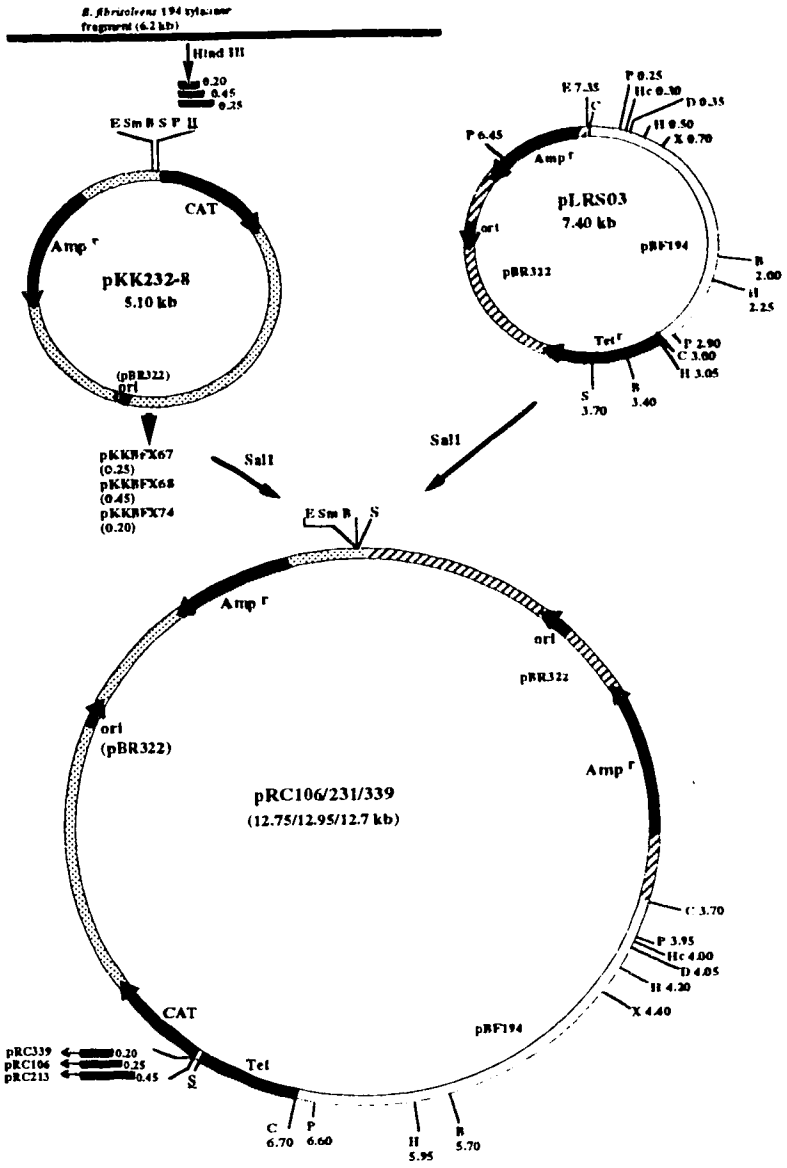

Figure 4.4. Schematic representation of the construction of the pRC shuttle vectors. The *B. fibrisolvens* 194 xylanase fragment (6.2 kb) was isolated from pBFX01, digested with HindIII, and the fragments cloned into the promoter probe vector, pKK232-8. pKK232-8, a pBR322 derivative, contains a promoterless chloramphenicol acetlytransferase gene (CAT) with a multiple cloning site, containing HindIII, inserted 5'-proximal to the CAT gene. Three HindIII fragments from the 194 xylanase gene were cloned into this site that initiated transcription of the CAT gene. These constructs, pKKBFX67, pKKBFX68, and pKKBFX74, were cloned into the SalI site of pLRS03 to yield pRC106, pRC213, and pRC339, respectively. Amp=ampicillin; Tet=tetracycline; ori=origin of replication; B=BamHI; C=ClaI; D=DraI; E=EcoRI; H=HindIII; Hc=HincII; P=PstI; S=SalI; Sm=SmaI; X=XbaI.

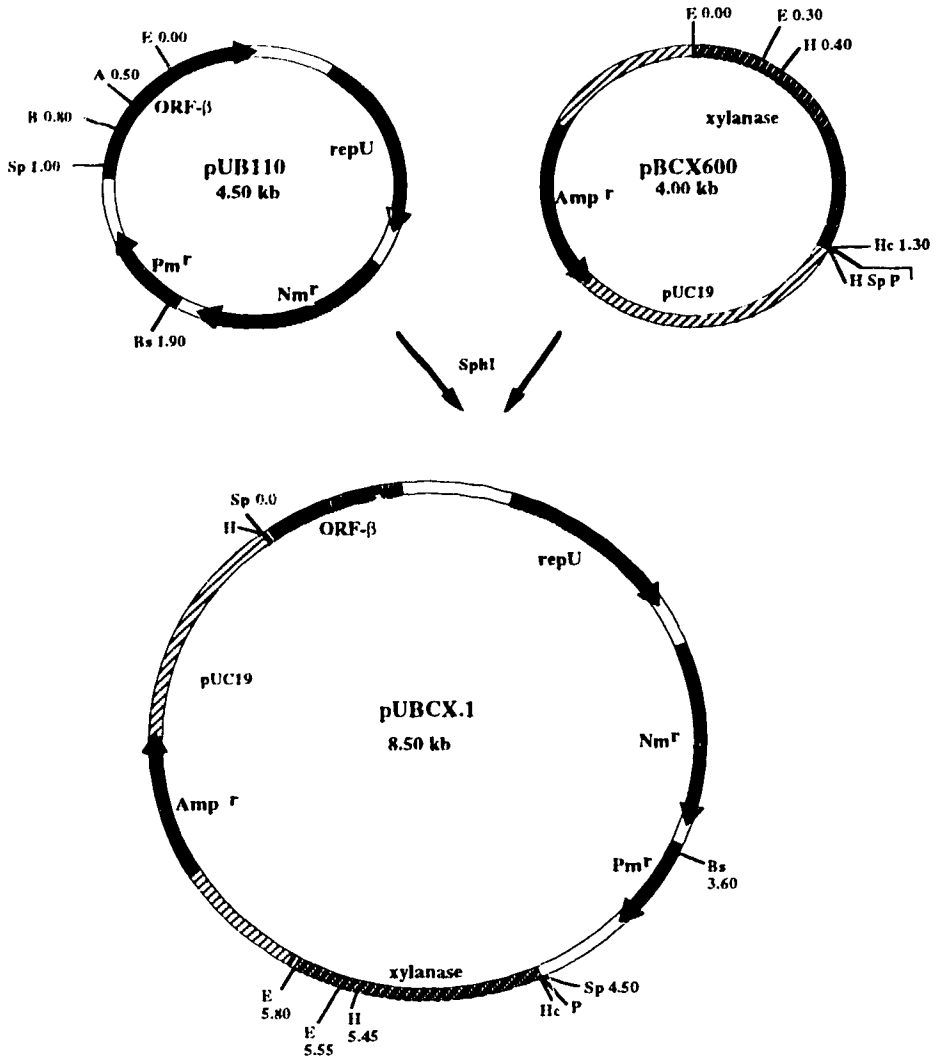

Figure 4.5. Schematic representation of the construction of pUBCX.1. pBCX600, which contains a 1.3 kb EcoRI-HincII *Bacillus circulans* xylanase fragment in pUC19, was cloned into the SphI site of the *Staphylococcus aureus* plasmid pUB110. The resulting vector, pUBCX.1, was non-mobilizable due to the interruption of ORF-ß and expressed ampicillin resistance (Amp^r), neomycin/kanamycin resistance (Nm^r), and phleomycin resistance (Pm^r). The vector also expressed xylanase activity in *E. coli*. ORF-ß=mobilization region; repU=replication origin; A=AvaI; Bs=BstXI; B=BamHI; E=EcoRI; H=HindIII; Hc=HincII; P=PstI; Sp=SphI.

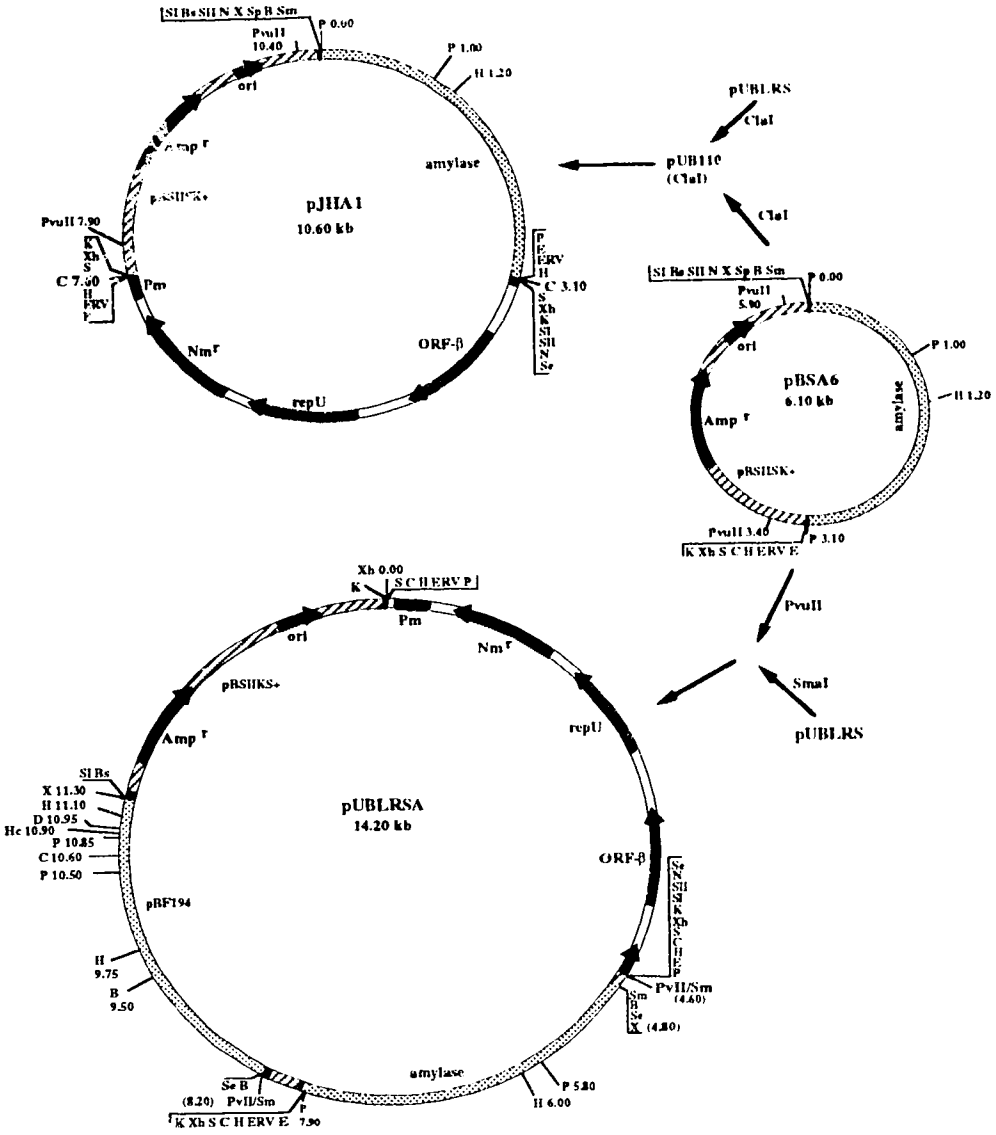

Figure 4.6. Construction of pJHA and pUBLRSA. To construct pJHA the *Streptococcus bovis* amylase gene was cloned from pSBA03 into the PstI site of pBluescriptIISK+ (pBSA6). Clones expressing amylase activity contained both PstI fragments, from pSBA03, in the orientation indicated. pUB110 was isolated from the vector pUBLRS (Clark et al., 1994) by ClaI digestion and cloned into the ClaI site of pBSA6, resulting in pJHA. The 3.6 kb PvuII amylase fragment from pBSA6 was cloned into the SmaI site of pUBLRS to yield pUBLRSA. Both clones expresssed amylase activity in *E. coli* which was detected according to the procedure outlined in Materials and Methods. A=AvaI; B=BamHI; Bs=BstXI; C=ClaI; D=DraI; E=EcoRI; ERV=EcoRV; H=HindIII; Hc=HincII; K=KpnI; N=NotI; P=PstI; S=SalI; SI=SstI; SII=SstII; Se=SpeI; Sm=SmaI; Sp=SphI; X=XbaI; Xh=XhoI.

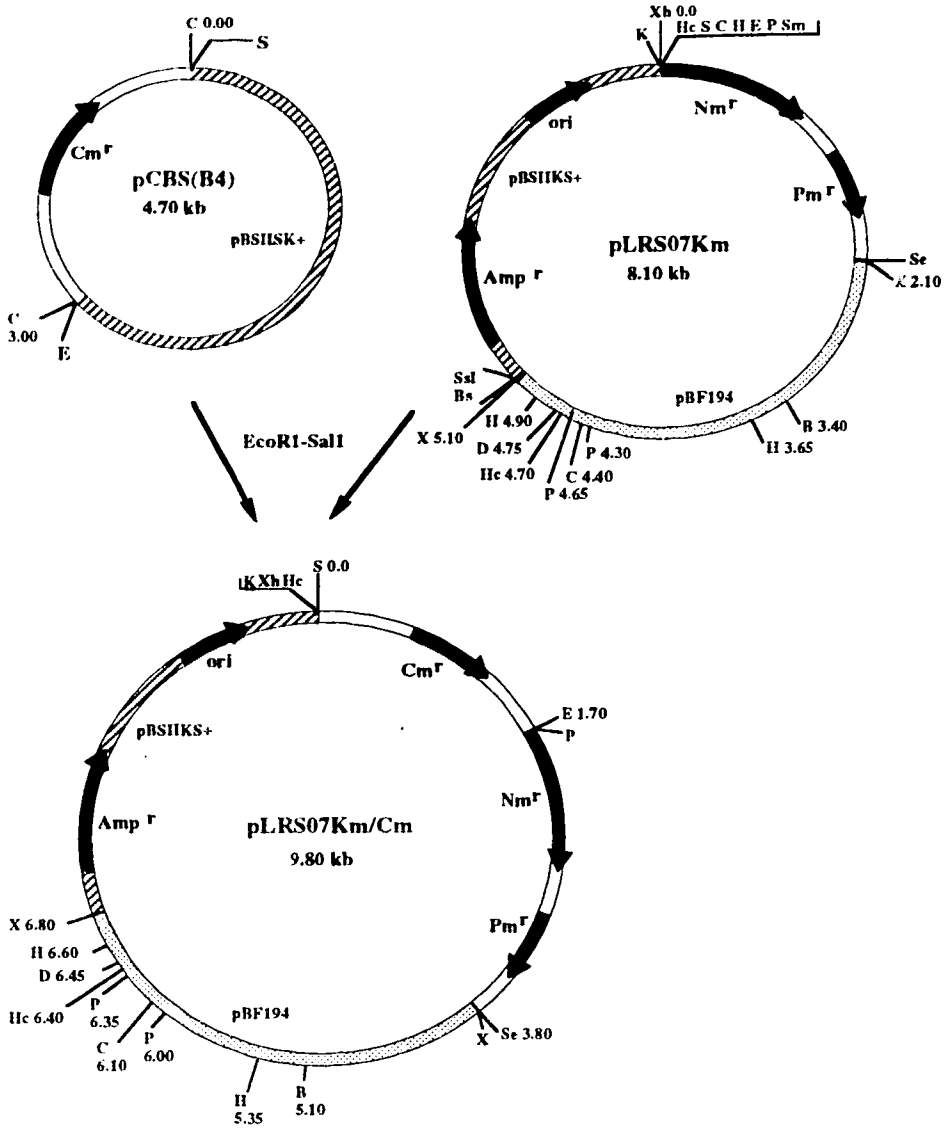

Figure 4.7. Cloning of the chloramphenicol gene (Cm) of the *Staphylococcus aureus* plasmid pC194 into the shuttle vector pLRS07Km. pC194 was digested with ClaI and the resulting fragments were cloned into pBluescriptIISK+. Clones containing the chloramphenicol gene expressed chloramphenicol resistance and these were screened for the number of fragments that had been cloned. pCBS4 contained only one copy of the 1.7 kb ClaI fragment containing the chloramphenicol resistance gene. The chloramphenicol gene was isolated from pCBS4 by EcoRI-SalI digestion and cloned into pLRS07Km. pLRS07Km/Cm expressed resistance to kanamycin (10 µg/ml) and chloramphenicol (10 µg/ml) in *E. coli*. B=BamHI; Bs=BstXI; C=ClaI; D=DraI; E=EcoRI; H=HindIII; Hc=HincII; K=KpnI; P=PstI; S=SalI; SI=SstI; Se=SpeI; Sm=SmaI; X=XbaI; Xh=XhoI.

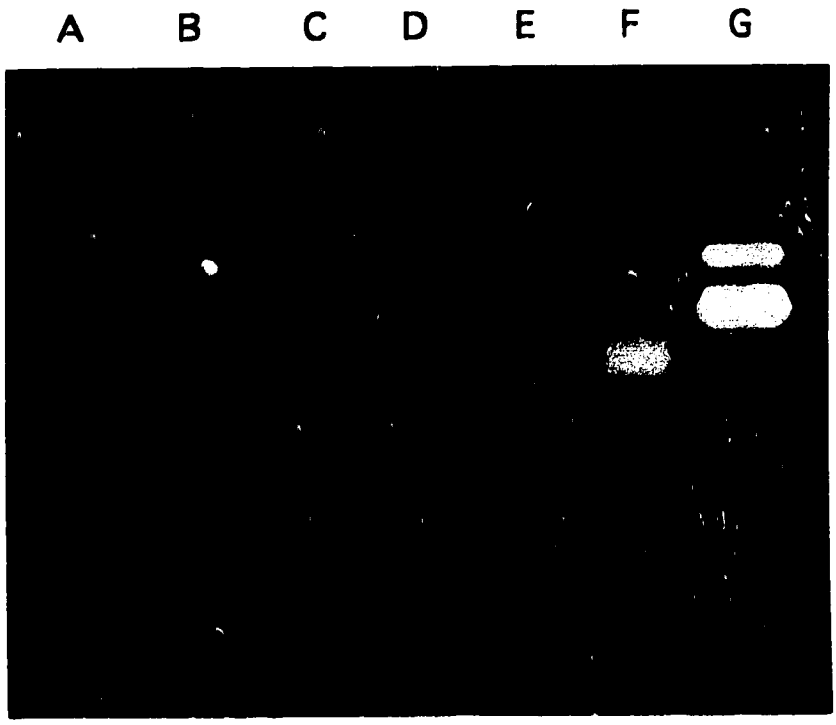

Figure 4.8. Zymogram analysis of H17c (pUBLRSA) transformants. The gel shows renaturation of amylase activity after SDS-PAGE and staining with iodine/potassium iodine solution (Materials and Methods). Starch was incorporated directly into the gels. The bands indicate the presence of amylase activity. (A) H17c grown on glucose [6.4], (B) H17c (pUBLRSA18) grown on glucose [5.0], (C) H17c grown on starch [4.1], (D) H17c (pUBLRSA18) grown on starch [2.7], (E) *S. bovis* 033 cell extract [9.5], (F) *S. bovis* 033 supernatant [0.51], (G) GM272 (pUBLRSA) [1.5]. The amount of protein loaded for each sample, in μg, is indicated in parentheses.

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5.0 A conjugative transfer system for the rumen bacterium, *Butyrivibrio fibrisolvens*, based on Tn*916*-mediated transfer of the *Staphylococcus aureus* plasmid pUB110.*

5.1 INTRODUCTION

Degradation of plant polysaccharides in the rumen is carried out largely by microbial digestion. A consortia of bacteria work to break down plant structural polysaccharides such as cellulose and hemicellulose. Although this process is relatively efficient it is not always complete. Conventional techniques, such as the chemical treatment of feedstuffs, have been studied as a means to improve the efficiency of feed utilization. The use of genetically modified species of rumen bacteria is another approach that could be used to improve digestion (Forsberg et al.,1986; Gilbert and Hazlewood, 1991; Forsberg and Cheng, 1992).

Butyrivibrio fibrisolvens is one of the predominant bacteria isolated from the rumen and seems a logical organism for genetic studies based on its characteristics. Most strains of *B. fibrisolvens* are highly xylanolytic, with many strains also digesting starch, lipids, proteins, pectin, and to some extent cellulose (Bryant and Small, 1956; Hespell, et al., 1988). This metabolic diversity means *B. fibrisolvens* is present in the rumen under a variety of feeding conditions. Cryptic plasmids have also been identified in some strains which could be used in the construction of vectors for *B. fibrisolvens* (Teather, 1982; Mann et al., 1986; Hefford, et al., 1993). Genes encoding for some of the amylases, xylanases, and cellulases

mediated transfer of the *Staphylococcus aureus* plasmid pUB110.*

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subtilis into *Bacillus thuringiensis*. Based on the study of Naglich and Andrews (1988), the ability of *B. fibrisolvens* H17c to be transformed using electroporation, and the fact that Tn*916* can be transferred into *B. fibrisolvens* we reasoned that mobilization of pUB110 may be possible between *B. fibrisolvens* strains. This paper describes the construction of two new pUB110-based shuttle vectors, pUBLRS and pLRS07Km, for *B. fibrisolvens*. Additionally, the development of *B. fibrisolvens* H17c donor strains which were used to mobilize pUB110 and pUBLRS into strains of *B. fibrisolvens* unable to be transformed using electroporation, is described.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are described in Tables 5.1 and 5.2. *Butyrivibrio fibrisolvens* strains were grown anaerobically at 39°C in RGM medium (Hespell et al., 1988) with 0.2% glucose (RGMG) or 0.4% arabinose (RGMA) as the carbon sources. *Ruminococcus spp.* were grown anaerobically at 39°C on the M2 medium of Hobson (1969) with 0.2% cellobiose as the carbon source. *Escherichia coli* strains and *Bacillus subtilis* BD366 were cultured on LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, 1 liter of distilled water) at 37°C. *Enterococcus faecalis* strains were cultured aerobically at 37°C on M17 medium (BDH Inc., Darmstadt, FRG) or anaerobically at 39°C on RGMG medium. Rifampicin or gentamycin resistant mutants were obtained by growing cultures to an approximate concentration of 10^8/ml, concentrating the cells in a volume of 1 ml, and plating 100 µl aliquots onto appropriate media containing rifampicin or gentamycin at concentrations from 1-5 µg/ml. Antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; chloramphenicol, 5-10 µg/ml; gentamycin, 5 µg/ml; kanamycin, 10-30 µg/ml; rifampicin, 5-30 µg/ml; and tetracycline, 10 µg/ml.

5.2.2 Electroporation experiments

B. fibrisolvens was transformed by electroporation according to the protocol of Whitehead (1992) with modifications to the protocol as follows. Cells were washed with10% polyethylene glycol (PEG; MW 8000) as the transformation efficiency could be

improved slightly using this buffer. A Gene Pulser Electroporation system was used (Bio-Rad Laboratories, Richmond, CA, USA) with 0.2 cm cuvettes and ... ieters of 2.0 kV, 25 μF, and 200Ω. Plasmid DNA used in the electroporation experiments was isolated from *E. coli* GM272.

5.2.3 Conjugation experiments

The conjugation protocol of Hespell and Whitehead (1991) was modified as follows with all procedures being done anaerobically in a glovebox with an atmosphere of 5% hydrogen-10% carbon dioxide-85% nitrogen. RGMG medium or M2-cellobiose medium (5 ml) in Hungate tubes was inoculated with overnight cultures (750 μl) and grown to a concentration of approximately 10^7-10^8 CFU/ml. Donor cultures were always grown in medium containing tetracycline (10 μg/ml) and kanamycin (20 μg/ml). The whole culture was centrifuged in the Hungate tubes at 3,000 rpm for 15 minutes at room temperature. Cell pellets were washed twice with 5 ml of RGM (no carbon source added) and resuspended in RGM (100 μl). Donor and recipient cells were mixed, vortexed, and placed on 25mm filters (0.22μm; Millipore Corporation, Bedford, MA, USA) on RGMG or M2-cellobiose plates and incubated at 39°C for approximately 12-16 hours. Control plates of separate donor and recipient cells were set up in the same way. After incubation the filters were placed in tubes containing 0.5 ml of RGM and cells removed by vortexing. Samples were plated on RGMG or M2-cellobiose selective plates and incubated for 2-7 days. Transfer frequencies were determined using the original cultures before washing. Counterselection of *E. faecalis*

in matings with *B. fibrisolvens* was done using modified RGM medium (RGM without yeast extract) with arabinose as the carbon source. *B. fibrisolvens* transconjugants were purified by plating three times on the selective medium and Gram stains performed to verify purity. Rifampicin or gentamycin was used for counterselection of the *B. fibrisolvens* H17c donor strain in matings with *B. fibrisolvens*, *R. albus*, or *R. flavefaciens* recipients.

5.2.4 DNA preparation and analysis

Plasmid DNA was prepared using a modified alkaline lysis procedure (Sambrook et al., 1989). Genomic DNA was isolated as described previously (Hu et al., 1991). Restriction enzyme digests were done according to procedures specified by the manufacturer (Gibco/BRL, Gaithersburg, MD, USA). DNA preparations were analyzed by electrophoresis on agarose gels (0.8% agarose in Tris-borate buffer [TBE; 0.089 M Tris, 0.089 M boric acid, 0.0025 M EDTA, pH 8.3]) run in TBE. DNA fragments were detected by staining gels in distilled water containing ethidium bromide (1 μg/ml) and visualized by UV transillumination.

The presence of plasmid DNA in *B. fibrisolvens* transformants and transconjugants was initially determined using a modified Eckhardt gel technique (Hynes et al., 1985). Cell pellets from100 μl of mid exponential *B. fibrisolvens* cultures were mixed with E1-lysozyme solution (10% sucrose, 100 μg/ml lysozyme, 10μg/ml RNase in TBE) and loaded directly on the gels or incubated for 15 minutes at 37°C before loading. Positive clones identified using this technique were analyzed further by hybridization with digoxigenin-

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5.3 RESULTS

5.3.1 Transformation of *B. fibrisolvens* by electroporation

Using electroporation *B. fibrisolvens* H17c was transformed with the *S. aureus* plasmid, pUB110, at an average frequency of 2.60 x 10^1 CFU/μg of DNA. Subsequently, two pUB110-based shuttle vectors, pLRS07Km and pUBLRS, were developed and used in electroporation experiments (Fig. 5.1). H17c transformants containing pLRS07Km occurred at a frequency of 5.00 x 10^1 CFU/μg of DNA. pUBLRS isolated from *E.coli* GM272 transformed H17c at a frequency of 3.28 x 10^2 CFU/μg in comparison to a frequency of 2.78 x 10^3 CFU/μg when pUBLRS was isolated from H17c. Resolution of plasmid DNA from select transformants on Eckhardt gels indicated the presence of these plasmids in H17c (data not shown). The Eckhardt procedure is useful for visualization of plasmids directly from cultures. Southern blot hybridization analysis of genomic DNA from transformants confirmed the presence of the appropriate plasmid in the transformants (data not shown). DNA of pUBLRS and pLRS07Km, isolated from both H17c and GM272, was digested and compared with no rearrangement or deletions of the plasmids evident in *B. fibrisolvens* (data not shown).

pUBLRS and pLRS07Km both contain a cryptic 3.0 kb plasmid, pBF194, which was isolated from *B. fibrisolvens* 194, a Svalbard reindeer strain, and characterized by restriction mapping (Fig. 5.1). In pLRS07Km the replication origin (repU) and ORF-β of pUB110 are absent and only the phleomycin and kanamycin/neomycin components

are present, while pUBLRS contains a complete copy of pUB110 (Fig. 5.1). The presence of pLRS07Km in H17c verifies that the pBF194 origin of replication is functional, as the pBSIISK+ origin of replication is not functional in *B. fibrisolvens.*

Although H17c could be transformed by electroporation this was not the case for *B. fibrisolvens* strains 193, 194, and 195. As pUB110 and pUBLRS could be introduced into H17c this made it possible to construct donor strains containing these plasmids and Tn*916,* as the mobilizing agent, to determine if pUB110 and the shuttle vector pUBLRS could be mobilized between *B. fibrisolvens* strains.

5.3.2 Construction of *B. fibrisolvens* H17c donor strains

Tn*916* was established in H17c (pUB110) and H17c (pUBLRS) via conjugation with *E. faecalis* CG110. Gram stains and comparison of genomic DNA digests to parental H17c and the respective transconjugant were used to verify the identity of the donor strains. Digoxigenin-labelled Tn*916*, digested with HindIII, was used to hybridize to HindIII digested genomic DNA of potential donor strains. Both H17c2 and H17c12 had two bands which hybridized indicating that only one copy of Tn*916* had inserted in the chromosome. Tn*916* has a single HindIII site which means two fragments are generated for every chromosomal copy of Tn916. These two donor strains were used in conjugation experiments with *B. fibrisolvens*, *R. albus*, and *R. flavefaciens* recipients.

5.3.3 Conjugative transfer of pUB110 and pUBLRS

B. fibrisolvens mutants resistant to rifampicin were mated

with the donor strains H17c2 and H17c12 which are sensitive to rifampicin. Screening of selected kanamycin/rifampicin resistant colonies revealed the presence of pUB110 or pUBLRS in the transconjugants. pUB110 was mobilized into *B. fibrisolvens* strains 193, 194, and 195. The shuttle vector, pUBLRS, was mobilized into *B. fibrisolvens* strains 193 and 194 when the recipients were heated, before mixing with the donor, at 45°C for 10 minutes. Average transfer frequencies are shown in Table 5.3. Mobilization of pUB110 into *R. albus* and *R. flavefaciens* did not occur in our experiments.

Several methods were used to verify that the putative *B. fibrisolvens* transconjugants were indeed the appropriate recipients containing pUB110 or pUBLRS. (i) Donor strain controls were treated the same way as the crosses in all matings and no or very low numbers of rifampicin/kanamycin mutants were detected. (ii) Colony morphologies were examined for the various recipients. (iii) *B. fibrisolvens*194 contains a distinct cryptic plasmid which could be visualized on Eckhardt gels to verify the identity of these transconjugants. Plasmid profiles have also proven useful in preliminary strain identification in *Rhizobium leguminosarum* (Hynes and O'Connell, 1990). (iv) Recipients were sensitive to tetracycline as Tn*916* is not transferred from H17c into other *B. fibrisolvens* strains (Hespell and Whitehead 1991). Tn*916* transconjugants were never detected when mating mixtures were plated on tetracycline/rifampicin plates. Additionally, all kanamycin resistant transconjugants or 50-100 from crosses with

numerous transconjugants were toothpicked onto RGM-glucose plates containing tetracycline. In all cases no growth was observed on tetracycline indicating that Tn*916* did not co-transfer with pUB110 or pUBLRS. (vi) Genomic DNA of selected transconjugants was digested with EcoR1 or HindIII and the digest patterns compared to the original recipients and the donor strain. Restriction patterns of *B. fibrisolvens* isolates show a high degree of variability which allows a qualitative comparison between strains (Hudman and Gregg, 1989). Using these techniques the putative transconjugants were identified as being the correct recipient strains. Once the identity of select transconjugants was verified they were analyzed by Southern blot hybridization using digoxigenin-labelled pUB110 or pUBLRS. pUB110 and pUBLRS could be identified in the recipient strains in this way (Fig. 2).

It was determined that Tn*916* is necessary for mobilization of pUB110 as crosses between the strain H17c(pUB110), missing Tn*916*, and the recipient *B. fibrisolvens* strains resulted in no rifampicin/kanamycin resistant transconjugants being detected.

5.3.4 Stability of plasmids in *B. fibrisolvens*

The stability of pUB110 in the transconjugants was determined by growth in nonselective medium. Select transconjugants were initially grown in RGMG containing kanamycin (20 μg/ml) and then subcultured into RGMG without antibiotics every 12 hours. Prior to transfer, cells were plated on RGMG plates with and without kanamycin. The cell counts on the kanamycin plates were compared with those on the non-selective medium to

determine the percentage of cells that maintained pUB110. For all transconjugants tested the kanamycin resistant phenotype rapidly declined from 100% of the population to 5-10% of the population. pUB110 was then maintained at these levels in subsequent passages through non-selective medium. pUB110 could be detected when selected kanamycin resistant colonies were analyzed on Eckhardt gels.

The stability of pUBLRS and pLRS07Km in *B. fibrisolvens* H17c was examined in the same way as pUB110. pUBLRS and pLRS07Km were maintained in approximately 55% of the total population. It was observed on Eckhardt gels that the presence of pUBLRS in 194 transconjugants resulted in the loss of the native plasmid pBF194. This incompatibility is not unexpected as the complete pBF194 replicon is present in pUBLRS.

5.4 DISCUSSION

Although it has been demonstrated that two *Butyrivibrio fibrisolvens* strains, H17c and AR10, can be transformed using electroporation (Ware, et al., 1992; Whitehead, 1992), we have been unsuccessful introducing plasmids into numerous strains of *B. fibrisolvens* by this method. Shuttle vectors developed in our lab, pUBLRS and pLRS07Km, could only be introduced into H17c. Guanine and cytosine (G + C) content, DNA cross-hybridization, and exopolysaccharide analysis have revealed that many of the strains identified as *B. fibrisolvens* may actually comprise as many as 24 different species (Mannarelli et al., 1990). This could explain why electroporation protocols developed for one strain, such as H17c, may not necessarily be appropriate for other strains of *B. fibrisolvens*. Additionally, most *B. fibrisolvens* strains produce extracellular polysaccharides that have varying degrees of viscosity (Stack, 1988; Ha, et al., 1991). It is possible that the higher viscosity exopolysaccharides produced by some strains would interfere with their ability to be transformed by electroporation. Modifications to our protocol such as varying the growth stage, electroporation parameters, buffers, and increasing the DNA concentration did not result in any transformants with strains 193, 194, and 195. Therefore, a conjugative transfer system was developed to facilitate the introduction of vectors into *B. fibrisolvens*.

In this study pUB110, a *Staphylococcus aureus* plasmid, was transferred into strains of *B. fibrisolvens* that were not transformed

by electroporation, using the conjugative transposon Tn*916* to mediate transfer. Transconjugants, containing pUB110, of strains 193, 194, and 195 were detected at average frequencies per donor of, 1.17 x 10^{-7}, 1.64 x 10^{-6}, and 5.76 x 10^{-7}, respectively. Transfer of pUB110 was dependent on the presence of Tn*916* as conjugation of pUB110 from H17c missing Tn*916* did not occur. Mobilization of pUB110 by the helper plasmid pLS20 and its derivatives requires an intact ORF-β (Selinger et al., 1990). No transconjugants have been detected in preliminary mobilization experiments with H17c donor strains containing pLRS07Km. This vector does not contain the ORF-β region of pUB110 and these results indicate that this region may also be used by Tn*916*.

pUBLRS was constructed for the purpose of being utilized as a mobilizable shuttle vector. Its ability to replicate in *E. coli* makes it easier to obtain DNA for cloning than for pUB110 which has to be propagated in *Bacillus subtilis*. Using electroporation this plasmid was introduced into *B.fibrisolvens* H17c. Significant amounts of this plasmid could be isolated from H17c as compared to pUB110, indicating that pUBLRS was maintained at a higher copy number than pUB110. pUBLRS could be mobilized from the donor strain H17c12, containing a chromosomal copy of Tn*916*, into *B. fibrisolvens* strains 193 and 194 at frequencies of 2.20 x 10^{-8} and 2.41 x 10^{-6} per donor, respectively. In initial experiments the mobilization of pUBLRS into *B. fibrisolvens* recipients was not detected. The protocol was modified by heat treating the recipients at 45°C for 10 minutes before mating to the donor strains. This modification resulted in

mobilization of pUBLRS. Heat treatment of the recipients may inactivate restriction endonucleases of the host which could interfere with transfer of a plasmid (Mazodier and Davies, 1991).

Hespell and Whitehead (1991) reported the inability of *B. fibrisolvens* to donate Tn*916* to other *B. fibrisolvens* strains and *E. faecalis* strains. In our experiments, tetracycline resistant transconjugants were not detected in experiments where mating mixtures were plated on rifampicin/tetracycline selective plates. Additionally, all kanamycin/rifampicin resistant transconjugants tested were tetracycline sensitive, indicating that Tn*916* was never co-transferred with pUB110 or pUBLRS. This phenomenon also has been observed in *Lactococcus lactis* (Bringel et al., 1992). Presumably a necessary host function, of unknown identity, is missing in these donor strains (Scott, 1992).

The ability to mobilize pUB110 and pUBLRS between strains of *B. fibrisolvens* extends the number of strains into which plasmid vectors can be introduced. The broad host range of pUB110 among Gram positive bacteria also suggests that mobilization into other Gram positive rumen bacteria is a possibility. Transfer of pUB110 into *Ruminococcus flavefaciens* and *R. albus* was not demonstrated in our study but this does not mean this transfer is not possible. Transfer may be occurring at a level not detected in our experiments and modifications to the procedure, as well as examining more *Ruminococcus* strains, may reveal that pUB110 can be mobilized into this organism.

pUBLRS and pLRS07Km will serve as useful shuttle vectors

that can be introduced into strain H17c by electroporation. The ability to mobilize pUBLRS means that it will be useful for *B. fibrisolvens* strains unable to be transformed using electroporation. The conjugation system described will facilitate the study of the expression and regulation of genes back in *B. fibrisolvens* which is an important aspect of developing genetically modified rumen bacteria.

*A version of this chapter has been accepted for publication. Clark et al., 1994. Plasmid, in press.

Table 5.1. Bacterial strains used.

Strains	Relevant characteristics	Reference
B. fibrisolvens		
H17c	nal^r str^r; steer isolate	Hespell and Whitehead (1991)
H17c2	Tn*916*; pUB110; nal^r kan^r tet^r	This study
H17c12	Tn*916*; pUBLRS; nal^r kan^r tet^r	This study
144	nal^r; steer isolate	LRSCC[a]
144R	rif^r mutant	This study
193	nal^r; Svalbard reindeer isolate	Orpin *et al.* (1985)
193R	rif^r mutant	This study
194	nal^r; Svalbard reindeer isolate	Orpin *et al.* (1985)
194R	rif^r mutant	This study
195	nal^r; Svalbard reindeer isolate	Orpin *et al.* (1985)
195R	rif^r mutant	This study
207	elk isolate	LRSCC
207R	rif^r mutant	This study
263	rif^r; moose isolate	LRSCC
R. flaveflaciens		
131	Svalbard reindeer isolate	Orpin *et al.* (1985)
131R	rif^r mutant	This study
363	tet^r str^r; bison isolate	LRSCC
363R	rif^r mutant	This study
R. albus		
016	str^r	Bryant *et al.* (1958)
016G	gm^r mutant	This study
196	Svalbard reindeer isolate	Orpin *et al.* (1985)
196G	gm^r mutant	This study
E. faecalis		
CG110	Tn916; tet^r rif^r fus^r	Franke and Clewell (1981)
B. subtilis		
BD366	pUB110;kan^r nm^r; *thr*-5 *trp*C2	BGSC[a]
E. coli		
DH5α	F- ϕ80d*lac*ZΔM15 *end*A1 *rec*A1 *thi*-1 *hsd*R17(r_k- m_k-) *sup*E44 λ- *gyr*A96 *rel*A1 Δ(*lac*ZYA-*arg*$_{\lambda}$,J159	BRL
DH10B	F- *mcr*A Δ(*mrr hsd*RMS *mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1*deo*R Δ(*ara, leu*)7697 *ara*D139*gal*U *gal*K *nup*G *rps*L	BRL
GM272	F- *hsd*S21 *dam*-3 *dcm*-6 *met*B1 *gal*K2 *gal*T22 *lac*Y1 *tsx*-78 *mtl*-1 *sup*E44 *thi*-1 *ton*A31	Marinus *et al.* (1983)

(a) LRSCC=LRS Culture Collection at Lethbridge Research Station, Lethbridge, Alberta, Canada. BGSC=Bacillus Genetic Stock Center
Abbreviations: gm=gentamycin; kan=kanamycin; nal=naladixic acid; rif=rifampicin; str=streptomycin; tet=tetracycline.

Table 5.2. Plasmids used in this study.

Plasmid	Relevant characteristics	Reference
pBluescriptII KS/SK+	amp^r; multipurpose cloning vector	Stratagene
pBSKm	amp^r kan^r; 2.1 kb Sau3A kan^r fragment of pUB110 cloned into BamH1 site of pBSIISK+	This study
pUB110	kan^r; Mob$^+$	Chopra *et al.* (1973) Gryczan *et al.* (1978)
pUB110exMCS	kan^r; Mob$^+$; contains MCS of pBSIISK+ in ThaI site of pUB110	Selinger (1993)
pBF194	cryptic *B. fibrisolvens* 194 plasmid	This study
pLRS07	amp^r; pBF194 cloned into XbaI site of pBSIIKS+	This study
pUBLRS	Mob$^+$; kan^r amp^r; pLRS07 cloned into XhoI site of pUB110exMCS	This study
pLRS07Km	amp^r kan^r; 1.8 kb XhoI-SpeI kan^r fragment of pBSKm cloned into pLRS07	This study

Abbreviations: Mob$^+$=mobilizable; MCS=multiple cloning site; amp=ampicillin; kan=kanamycin

Table 5.3. Frequency of transfer of pUB110 and pUBLRS into*B. fibrisolvens, R. flavefaciens* and *R. albus* strains from the donor strains *B. fibrisolvens* H17c2 and H17c12[c].

Recipient	Donor	Transfer frequency [a] per donor	per recipient
B. fibrisolvens			
144R	H17c2	0[b]	0
193R	H17c2	1.17×10^{-7}	1.33×10^{-7}
	H17c12	2.20×10^{-8}	4.81×10^{-9}
194R	H17c2	1.64×10^{-6}	2.29×10^{-5}
	H17c12	2.41×10^{-6}	9.10×10^{-8}
195R	H17c2	5.76×10^{-7}	1.02×10^{-5}
207R	H17c2	0	0
263	H17c2	0	0
R. flaveflaciens			
131R	H17c2	0	0
363R	H17c2	0	0
R. albus			
016G	H17c2	0	0
196G	H17c2	0	0

(a) Average transfer frequencies based on 2 - 4 replicates
(b) no transconjugants detected; frequency $<10^{-9}$
(c) selection for transconjugants was done on $kan_{30}rif_{10}$ or $kan_{30}gm_{10}$

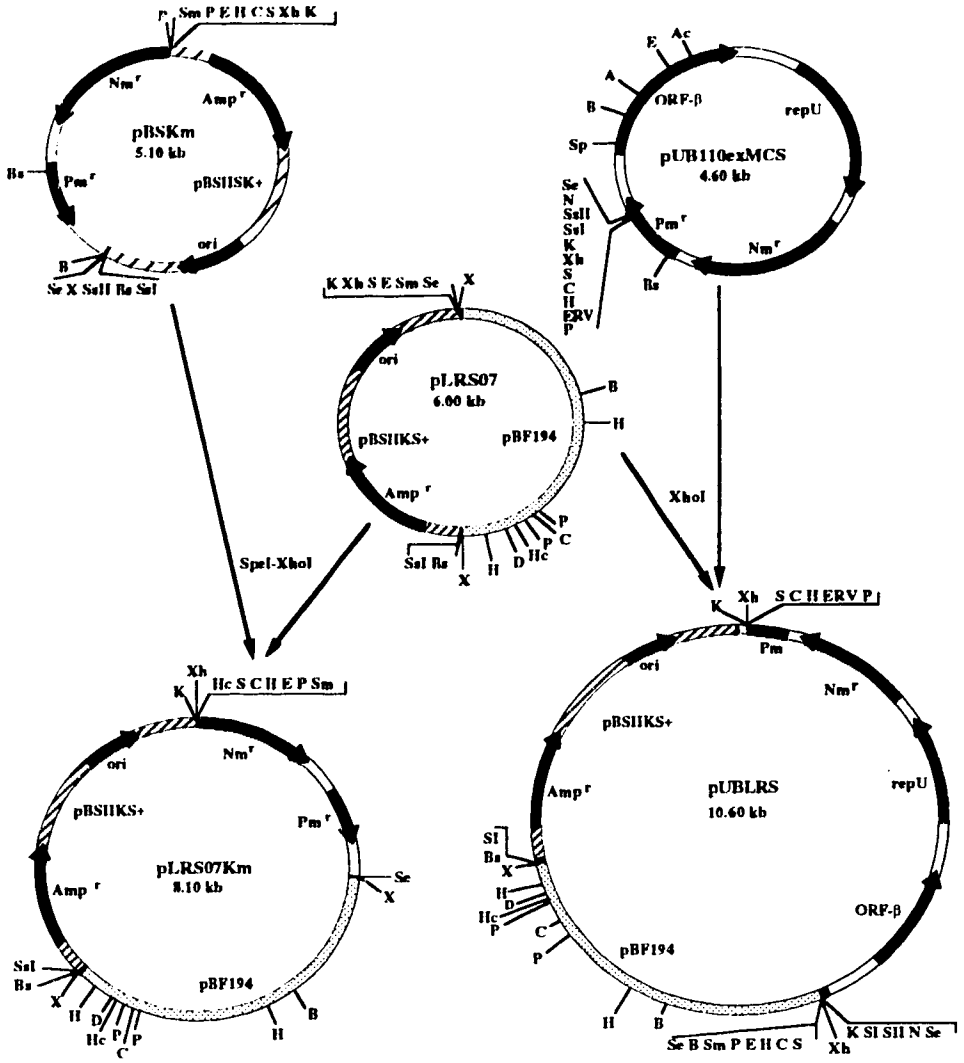

Figure 5.1. Construction of the electroporation vectors pUBLRS and pLRS07Km. To construct pUBLRS pUB110exMCS was cloned into the Xho1 site of pLRS07. pUBLRS contains an intact ORF-ß and the phleomycin (Pm) resistance gene has been inactivated but the neomycin/kanamycin (Nm) and ampicillin resistance genes are still intact. The potential unique cloning sites in pUBLRS are Spe1 and Sma1. The electroporation vector pLRS07Km contains the kanamycin gene of pUB110. A 2.1 Kb Sau3A fragment containing the neomycin/kanamycin (Nm) and phleomycin (Pm) resistance genes from pUB110 was first cloned into the BamH1 site of pBluescriptIISK+ to give the intermediate vector pBSKm. The 2.1 Kb Spe1-Xho1 fragment of pBSKm, containing the Nm and Pm genes, was then cloned into pLRS07. The unique cloning sites in this vector are EcoR1 and Sal1. pBF194 is a cryptic B. fibrisolvens plasmid that was cloned into pBluescriptIIKS+ (pLRS07). A=Ava1; Ac=Acc1; B=BamH1; Bs=BstX1; C=Cla1; D=Dra1; E=EcoR1; ERV=EcoRV; H=HindIII; Hc=HincII; K=Kpn1; N=Not1; P=Pst1; S=Sal1; Sc=Spe1; Sm=Sma1; Sp=Sph1; SsI=SstI; SsII=SstII; X=Xba1; Xh=Xho1.

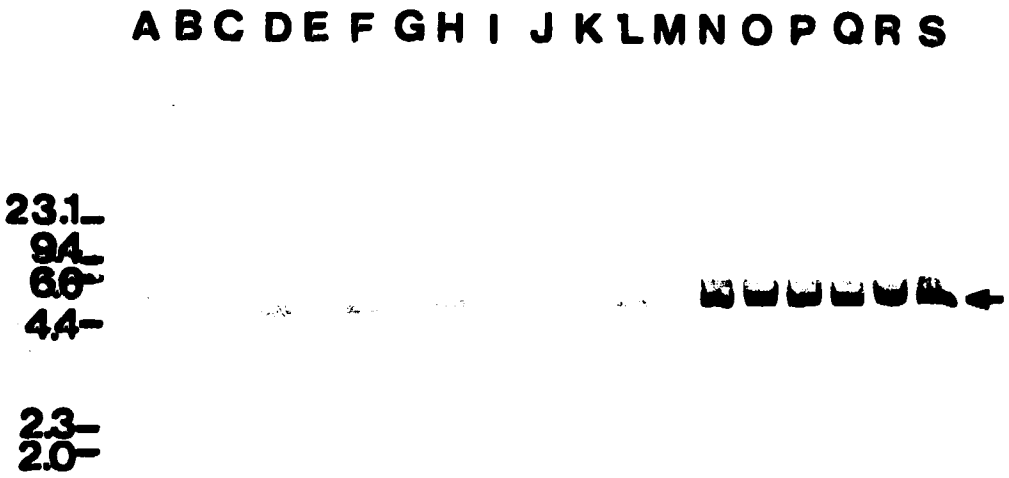

Figure 5.2. Southern blot hybridization of XbaI digested genomic DNA from *B. fibrisolvens* 193, 194, and 195 transconjugants using random primed digoxigenin-labelled pUB110. Lanes contain, (A) H17c2 donor; (B) 193 recipient; (C-F) 193 (pUB110) transconjugants; (G) 194 recipient; (H-L) 194 (pUB110) transconjugants; (M) 195 recipient; (N-R) 195 (pUB110) transconjugants; (S) pUB110 (XbaI). Molecular weights were estimated by comparison with a HindIII digest of lambda DNA (sizes indicated on side of blot). Linear pUB110 is indicated by an arrow.

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6.0 CONCLUSIONS AND GENERAL DISCUSSION

6.1 Project summary

In this project the following has been achieved:

1. An amylase gene from *Streptococcus bovis* strain 033 was cloned and studied in *Escherichia coli.* The amylase gene was subcloned on a 3.1 kb fragment and Tn*5* mutagenesis indicated that the entire gene, including its promoter, had been cloned. This *S. bovis* 033 amylase did not hybridize to genomic DNA of the *S. bovis* 077 type strain or to genomic DNA of *Butyrivibrio fibrisolvens* strains194 and 195. The 033 amylase gene was eventually cloned into shuttle vectors developed during the project and introduced into *B. fibrisolvens* H17c.

2. Numerous vectors were developed based on the cryptic *B. fibrisolvens* plasmid pBF194 and many Gram negative and Gram positive vectors. All of these vectors were studied in *E. coli* but many of them were never successfully introduced into *B. fibrisolvens.* The *Staphylococcus aureus* plasmid pUB110 and the pUB110-based vectors, pUBLRS and pLRS07Km, were introduced into *B. fibrisolvens.*

3. Electroporation was used to introduce pUB110, pUBLRS, and pLRS07Km into strain H17c. These vectors were stably maintained in *B. fibrisolvens* and conferred resistance to kanamycin. Strains 193, 194, and 195 were never transformed using the technique of electroporation.

4. H17c donor strains were developed that contained Tn*916*, a conjugative transposon donated from *Enterococcus faecalis,* and

either pUB110 (H17c2) or pUBLRS (H17c12). pUB110 was mobilized from H17c2 into *B. fibrisolvens* strains 193, 194, and 195 using Tn*916* as the mobilizing agent. pUBLRS was mobilized from H17c12 into *B. fibrisolvens* 193 and 194. Heat treatment of the recipients was required in order to detect transfer of pUBLRS. pUB110 was not mobilized into *Ruminococcus flavefaciens* or *R. albus.*

5. Foreign genes were cloned into pUBLRS and pLRS07Km to demonstrate their usefulness as shuttle vectors. The amylase gene from *S. bovis* 033 was cloned into pUBLRS (pUBLRSA) and the chloramphenicol gene from the *S. aureus* plasmid pC194 was cloned into pLRS07Km (pLRS07Km/Cm). Both of these constructs were introduced, by electroporation, into *B. fibrisolvens* H17c. The presence of pLRS07Km/Cm in H17c conferred resistance to 5 μg/ml of chloramphenicol. Analysis of pUBLRSA transformants on SDS-polyacrylamide gels revealed bands of amylase activity which differed from those in wild type H17c. These results indicate that proteins encoded by genes introduced into *B. fibrisolvens* H17c can be expressed.

The focus at the beginning of my project was to develop an electroporation protocol that could be used to introduce constructed vectors into strains of *B. fibrisolvens.* During the course of this work Ware *et al.* (1992) and Whitehead (1992) were successful in transforming *B. fibrisolvens* strains AR10 and H17c, respectively, using electroporation. Using the protocol of Whitehead (1992) I introduced the *S. aureus* plasmid pUB110 and two pUB110-based vectors I constructed, pUBLRS and pLRS07Km, into strain H17c.

Electroporation only worked with strains H17c and not with other strains used in this project. Numerous modifications to the protocol did not result in transformation of these strains. However, the Tn*916*-mediated conjugation system was developed which resulted in the transformation of those strains which had proven recalcitrant to electroporation. This is a novel conjugation system for *B. fibrisolvens* as it uses conjugation to mobilize a shuttle vector into strains which could not be transformed using other methods. The *S. bovis* amylase and a chloramphenicol resistance gene were cloned into shuttle vectors and introduced into strain H17c. This is the first time a polysaccharidase from another rumen bacterium has been introduced and expressed in *B. fibrisolvens.*

The vectors and transfer systems described represent an advancement in the study of the molecular biology of *B. fibrisolvens.* It will be interesting to attempt to introduce other polysaccharidases, such as xylanases and cellulases, into *B. fibrisolvens* using these systems. Future work should concentrate on analyzing the effect which expression of foreign genes has on the metabolic activity of *B. fibrisolvens.* It will also be crucial that the effect which genetically modified bacteria have on the function of a multi-species consortium be determined.

6.2 Future of molecular biology in rumen microbiology research

The cloning of genes from rumen bacteria has helped elucidate the numbers of enzymes present in some of the rumen bacteria that

are involved in the digestion of plant polysaccharides. With the development of vectors and transfer systems for rumen bacteria, how these enzymes function and how many are required in digestion will be revealed. Our basic understanding of the genetic basis of metabolic activities of rumen bacteria will be enhanced through molecular biology techniques.

Ruminants rely on communal processes where a consortium of microorganisms are responsible for the digestion of feedstuffs. I have concerns with developing a genetically modified rumen bacterium based on strains maintained as pure cultures in the laboratory over a number of years. Have our studies become too focused on laboratory cultured strains? It is conceivable that work done on pure cultures will not reflect events in the natural environment of the bacterium; the rumen. Are we creating, as Woese (1994) has stated, a zoo of monsters, laboratory freaks that perform the functions we require of them but cannot exist back in their original niche. It may turn out that laboratory strains, modified for improved fiber digestion, may not be suitable for introduction into the rumen. A study by Attwood et al. (1988) found that an introduced laboratory strain of *Prevotella ruminicola* was rapidly lost from the rumen. Possibly we should concentrate on using fresh rumen isolates of bacteria, rather than long-term laboratory cultured strains, when developing genetic techniques (Hudman and Gregg, 1989). Although we have begun to understand the genetics of individual laboratory strains through these molecular studies, will we have an appreciation for the complexity of interactions in the

rumen ecosystem?

During the course of my work I have observed how diverse strains of a given species can be. Genetic techniques developed for one strain will not work for other strains of the same species. This is not a new concept! The diversity within a species of rumen bacteria has been observed and studied by a number of research groups. Genetic diversity within *Selenomonas ruminantium, B. fibrisolvens, Fibrobacter succinogenes,* and *P. ruminicola* has been revealed through the use of molecular biology techniques (Mannarelli, 1988; Mannarelli et al., 1990; Mannarelli et al., 1991; Ning et al., 1991). DNA hybridization studies and exopolysaccharide analysis of strains identified as *B. fibrisolvens* has resulted in their division into five groups, some which may represent new species of *Butyrivibrio* (Mannarelli, 1988; Mannarelli et al., 1990). Although there is genetic diversity of species within a genus there can still be a phylogenetic relationship. *F. succinogenes,* originally classified as *Bacteroides,* has been divided into different species but is phylogenetically coherent, with all members performing the common function of cellulose digestion (Amann et al., 1992). This diversity of species should be appreciated and taken into account when developing molecular techniques for the rumen bacteria. Unfortunately, in many cases, we choose to ignore this diversity and forget that it is relevant to our work.

Molecular biology can contribute more than just an understanding of the genetic regulation of fiber digesiton in individual bacteria. As mentioned previously, molecular biology

techniques can be used to identify the genetic diversity of species within a genus. This will lead to an understanding of the phylogeny of these bacteria and how they evolved to occupy their particular niche in the rumen.

Molecular biology techniques will also allow a comprehensive study of rumen microbial populations in their natural ecosystem. DNA probes based on 16S rRNA sequences have already been used to study the effects of dietary changes or the addition of the ionophore monenesin on selected bacterial strains in the rumen (Stahl et al., 1988; Briesacher et al., 1992). These types of studies have generally been avoided due to the fact that they are very tedious and time consuming. It is studies such as these that will provide valuable information on the microbial diversity and the role of all rumen microorganisms in the rumen. These types of molecular studies of rumen ecology may also reveal the presence of additional microbial species, which were never identified because they could not be isolated and cultured in the laboratory.

Researchers involved in genetic studies must keep in perspective the diversity that exists in the rumen ecosystem and the balance that has been achieved with microbe-microbe and microbe-animal interactions. We must not lose sight of how efficient ruminants are at converting feeds, inaccessible for use by non-ruminants, into energy for their use. Most importantly we must not forget that it is the minute prokaryote that makes this possible. Prokaryotes underlie all living processes and are the real chemists of this planet (Woese, 1994).

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