16S Ribosomal DNA Probes for the Detection and Enumeration of Proteolytic Rumen Bacteria

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ABSTRACT

Bacterial degradation of protein causes inefficient nitrogen retention in New Zealand ruminants. The 16S rRNA genes of a *Butyrivibrio fibrisolvens*-like strain and three *Streptococcus bovis* strains, isolated from New Zealand cattle were sequenced to further characterise these isolates. Based on 16S rDNA analysis the *B. fibrisolvens*-like isolate was classified as *Clostridium proteoclasticum*, while the three *S. bovis* isolates were confirmed as *S. bovis* strains.

In the absence of selective media for enumeration of these bacteria, a competitive PCR (cPCR) approach was developed for enumeration of these bacteria from rumen samples. PCR primers were designed to variable regions within the 16S ribosomal RNA genes of both *S. bovis* and *C. proteoclasticum.* These primers were used in conjunction with the universal forward primer fD1*, to allow amplification of 16S rDNA fragments from these organisms. DNA database searches revealed that the B316 830 primer sequence was present in four *B. fibrisolvens* strains. Analysis of 16S rDNA sequences indicated that these *B. fibrisolvens* strains are closely related to *C. proteoclasticum* and that the B316 830 primer circumscribes these five strains.. The B315 454 primer sequence was found in the 16S rDNA of 10 *Streptococcus* species. Primer specificity was tested in amplification reactions with DNA extracted from 85 bacterial isolates, mainly of rumen origin. The *C. proteoclasticum* primer B316 830 and fD1* produced a specific PCR product from *C. proteoclasticum* DNA only, while the *S. bovis* primer B315 454 and fD1* gave specific PCR product from DNA of all strains of *S. bovis* tested but from no other rumen bacterium. An internal control was developed for both *S. bovis* and *C. proteoclasticum* to use in cPCR reactions for quantitation. Standard curves were constructed relating the PCR product intensity of target DNA extracted from a known number of cells and the intensity of internal control DNA PCR product. The standard curves were used to quantitate populations of *S. bovis* and *C. proteoclasticum* in rumen samples collected from eight dairy cows fed a rotation of four diets. Populations detected ranged from 2×10^6 to 2.8×10^7 for *C. proteoclasticum* and 1.7×10^7 to 1.3×10^8 for *S. bovis.* Diet had no significant effect on the populations of either of these proteolytic bacteria.

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Chapter 1 - Introduction and Literature Review

1.1 Introduction

Ruminants are important to New Zealand's economy, contributing fibre, meat and dairy products for the export market. Despite this importance, little is known about the rumen microflora that inhabit the New Zealand ruminant. These microbes are responsible for the conversion of pasture to products for utilization by the ruminant. Pasture consumed by the ruminant enters the rumen, where a combination of muscular movements, remastication and microbial digestion break the plant material down into compounds that are beneficial for both the microbial community and the ruminant.

The rumen microbial population consists of bacteria, protozoa and fungi. These organisms exist in a symbiotic relationship with the ruminant, where the animal provides controlled pH, temperature, and a steady supply of fermentable substrates. In return the microorganisms breakdown the plant material to volatile fatty acids, mainly butyrate, propionate and acetate, which are utilized by the animal as a source of energy. Plant protein is converted mainly to microbial protein, which provides the major nitrogen supply for the animal, as organisms are flushed from the rumen and are hydrolysed in the abomasum (true stomach). Bacteria provide the greatest source of microbial protein to the animal, while protozoa are largely retained in the rumen (Williams and Coleman, 1988).

The rumen originally evolved for the digestion of the fibrous plant material that constituted the diet of the early ruminant. The large volume of the rumen, along with remastication by the animal and increased retention times contributed to improved digestion efficiency. The rumen microbial population has also evolved largely to digest the cellulose and hemi-cellulose of the ruminant diet. However, with the domestication of ruminants and the subsequent development of intensive pastoral practices there has been an increasing requirement for improved efficiency of growth and production by the animal for better economic returns. Pastures grazed by ruminants in New Zealand are high in protein and low in soluble sugars (Johns, 1955). Plant protein in the rumen is rapidly degraded, mainly by bacteria (Brock *et al.*, 1982) to ammonia and the amount of ammonia released in the rumen often exceeds the amount which the microbial

population can utilize . The excess ammonia is adsorbed across the rumen epithelium and converted to urea in the liver and excreted from the kidneys as urine (Nolan , 1975). This represents a net waste of nitrogen from the animal.

The problem of improving nitrogen utilization in the New Zealand ruminant is important to the industries that rely on ruminant products, as efficient nitrogen utilization by the animal would lead to a reduction in input costs and an increase in profits. An understanding of protein degradation in the ruminant and the microorganisms involved is required so that strategies for improvement of nitrogen metabolism in ruminants can be formulated.

1.2 NITROGEN METABOLISM IN THE RUMEN.

Many sources of nitrogen enter the rumen including nitrates, nitrites, nucleic acids, urea and mucosal proteins, but the majority is in the form of feed protein. Most of this protein is degraded in the rumen, but some passes through the rumen for digestion in the abomasum. Degradation of feed protein in the rumen is performed by bacteria, fungi and protozoa. Bacteria have the highest specific proteolytic activity of the rumen microflora, and are thought to be the major digesters of protein (Brock *et al.,* 1982). Between 30 and 50 percent of rumen bacteria have proteolytic activity towards extracellular protein (Fulghum and Moore, 1963; Prins *et al.*, 1983) and members of the genera *Prevotella, Butyrivibrio, Streptococcus, Bacillus, Selenomonas, Succinivibrio, Eubacterium, Lachnospira* and *Clostridium* have been identified as being important in dietary protein breakdown (Fulghum and Moore, 1963; Brock *et al.,* 1982; Wallace and Brammall, 1985).

Proteins are broken down by mainly cell associated proteinases and peptidases (Blackburn and Hobson, 1960; Kopecny and Wallace, 1982; Prins *et al.,* 1983). The predominant proteinase activity within ruminant fed a dried forage and concentrate diet is a cysteine type protease (Wallace and Brammall, 1985). Recently it has been proposed that *Prevotella ruminicola* be reclassified into four new species, *P. ruminicola, P. bryantii, P. brevis and P. albensis* (Avgustin *et al.,* 1997). These bacteria have a proteolytic activity that is most similar to that of the bacterial fraction (Wallace and Brammall, 1985), and on dried forage and concentrate diet they are probably the most important proteolytic organisms. Their proteolytic properties are also most similar to that of mixed rumen contents (Wallace and Cotta, 1987), but the contribution of each of the individual *Prevotella* species makes to protein degradation is not known. Isolates of *Ruminobacter amylophilus, Butyrivibrio fibrisolvens* and *Fusobacterium* sp. have been found to have high proteolytic activity (Wallace and Brammall, 1985), but their activities were of the serine proteinase type, which is not the prevalent proteolytic activity of the mixed bacterial population (Wallace and Brammall, 1985). *Streptococcus bovis* is another potentially important proteolytic bacterium, with a low proteolytic activity, but very high leucine aminopeptidase activity, and probably contributes very actively to exopeptidase activity in the rumen (Wallace and Bramwell, 1985). The products of protein breakdown in the rumen are oligopeptides, peptides and

amino acids. These are either utilized by bacteria or degraded further by deaminases to short chain fatty acids and ammonia. Dipeptides are cleaved off oligopeptide chains by dipeptidyl peptidases produced mainly by *P. ruminicola* (Wallace *et al.*, 1990), followed by cleavage into amino acids by dipeptidases, the predominant form of amino peptidase in the rumen fraction.

Until recently reports had indicated that peptides were the preferred form of amino acids for incorporation into protein by the mixed bacterial fraction protein and that this was the most efficient method of assimilation (Wright, 1967; Wright and Hungate 1967; Prins 1977). However, Ling and Armstead (1995) have shown that *S. bovis, S. ruminantium, F. succinogenes, and Anaerovibrio* uptake free amino acids in preference to peptides as an amino acid source, while only *P. ruminicola* preferred peptides. Therefore the observation that the bacterial population has a preference for peptide uptake was probably a reflection of the predominance of *P. ruminicola* in the population (Wallace *et al.,* 1997).

In the rumen the majority of free amino acids are not transported across the bacterial cell wall for direct incorporation into microbial protein, but are deaminiated, mainly by ciliate protozoa (Hino and Russell, 1985), leaving carbon skeletons for energy production and ammonia. Therefore the majority of rumen bacteria do not utilise amino acids as a source of energy. However inconsistencies in the amounts of ammonia produced in the rumen and the amounts produced by common rumen bacteria led to the discovery of the hyper-ammonia producing bacteria (Chen and Russell, 1988; Russell *et al.,* 1988; Chen and Russell, 1989). Three bacteria, *Clostridium sticklandii, Clostridium aminophilum* and *Petostreptococcus anaerobius* were discovered that produce large amounts of ammonia from the fermentation of peptides and amino acids (Chen and Russell, 1988; Russell *et al.,* 1988; Chen and Russell, 1989). U.S. studies have shown the combined activities of these bacteria may account for a significant amount of ammonia production in the rumen (Chen and Russell, 1988; Russell *et al.,* 1988; Chen and Russell, 1989).

Ammonia produced from deamination in the rumen is reassimilated by rumen bacteria to synthesize amino acids which are eventually incorporated into bacterial protein (Hespell, 1984). Between 50 and 78% of the ammonia is taken up by the bacterial cell

is converted into amino acids (Hespell, 1984). Assimilation can occur by several mechanisms. The glutamine-synthetase/glutamate-synthase (GS-GOGAT) couple has the highest affinity for ammonia, where ammonia is added to glutamate to form glutamine by glutamine synthetase. The $-NH_3$ is then transferred to α-oxoglutarate, forming two glutamate molecules. However, the low levels of GOGAT detected in the rumen and the fact that it requires ATP, indicate that this system is used when ammonia concentrations are low (Wallace and Cotta, 1987). The most likely alternative enzyme for ammonia assimilation is NADH-glutamate dehydrogenase (NAD-GDH), which has the highest activity of ammonia assimilation in rumen contents and in rumen mucosa. Other possible enzymes include NADP-GDH, alanine dehydrogenase, asparagine synthetase, NADP-alanine dehydrogenase and aspartate dehydrogenase (Hespell, 1984). Once the ammonia is fixed to a primary amino acid, aminotransferases transfer the $-NH_2$ group to other amino acids within the intracellular amino acid pool.

However the rate of amino acid assimilation by rumen microbes is far less than the rate of ammonia production, due to the lack of synchrony in plant fibre degradation in the rumen. The plant protein released by mastication of the feed is broken down rapidly in the rumen, as are the soluble polysaccharides, but the more recalcitrant plant components such as cellulose and hemicellulose are degraded at a much slower rate, limiting the amount of energy available for ammonia assimilation. Excess ammonia diffuses across the rumen epithelium and transported to the liver by the bloodstream, where it is converted to urea. Some of this urea is recycled back to the ruminant through saliva and diffusion through the rumen wall, but the majority is transported to the kidneys for excretion (Nolan, 1975).

Most of the information available on rumen nitrogen metabolism comes from studies done in the Northern Hemisphere on animals grazing dried forage and concentrate diets. There is little corresponding information from animals grazing fresh forage under New Zealand conditions. Pastures grazed by New Zealand ruminants contain high amounts of protein and low amounts of soluble carbohydrates (Johns, 1955). Studies of the proteolytic rumen bacteria from New Zealand cattle (Attwood and Reilly, 1995) have shown that *Streptococcus bovis* is the predominant proteolytic organism, comprising 61% of the proteolytic isolates. *Eubacterium* sp. and *Butyrivibrio* sp. were also observed. In contrast to studies on Northern Hemisphere animals, *Prevotella*

ruminicola isolates were not common and only one isolate was described as *Prevotella* like. The specific proteolytic activities of the New Zealand isolates also differed from the Northern hemisphere activities. The mixed microbial population had a predominately cysteine proteolytic activity, as did the Northern hemisphere animals (Attwood and Reilly 1996), but the highest specific activity was a N-succinyl Ala Ala Pro Phe *p*-nitroanilide (NSAAPPPNA), chymotrypsin like activity in the New Zealand mixed microbial fraction, while in previously studied bacterial fractions benzoyl arginine *p*-nitroanalide (BAPNA) was the main activity (Wallace and Bramwell, 1985). Strain C21a, the *Prevotella*-like isolate, did not have a proteolytic activity that matched the mixed microbial fraction, whereas *Prevotella* dominated the bacterial population in Northern Hemisphere ruminants, and hence influenced the proteolytic activity of the mixed fraction. Nor did other isolates tested match the mixed microbial fraction, though *S. bovis* strain B315 proteinase activity was similar (Attwood and Reilly, 1996). A novel bacterium, strain B316, produced the highest proteinase activity, and was later described as *Clostridium proteoclasticum* (Attwood *et al.*, 1996).

It is apparent that proteolytic populations of bacteria in New Zealand ruminants differ from the previously intensely studied Northern Hemisphere proteolytic populations. With the selection pressures of artificial culturing it is difficult to accurately enumerate bacteria using classical techniques. Therefore the major contributors to rumen proteolysis under New Zealand conditions need to be investigated further to positively identify them and to determine numbers *in vivo*, so that the contribution of these bacteria to rumen proteolysis under New Zealand feeding regimes can be properly assessed.

1.3 IDENTIFICATION AND CLASSIFICATION OF BACTERIA IN THE RUMEN

The rumen contains an extremely diverse microbial ecosystem, which includes many bacterial species performing a multitude of reactions. The identification and classification of these bacteria is an important step in describing the biodiversity of microorganisms in this environment. Accurate descriptions are also essential for future reference and accurate phylogenetic inferences. In classical taxonomy descriptions of animal and plants are based on morphological features. However the limited number of possible bacterial morphotypes does not provide a reliable form of classification for these organisms. In the past, classical techniques including cell staining, carbon source utilization, antibiotic resistance and cell morphology, have been used to identify bacterial isolates. These techniques require isolation and culture of organisms, placing artificial pressures upon their growth and natural behavior. This is particularly relevant to rumen microbiology as replicating anaerobic conditions that mimic the rumen environment is extremely difficult. As a result, several species of rumen bacteria have been misidentified and have now been assigned to new genera (Flint *et al.*, 1990; Mannarelli *et al.*, 1990; Shah and Collins, 1990; Mannarelli *et al.*, 1991).

1.3.1 Molecular methods for phylogenetic analysis.

Classifications based on bacterial phenotypes are limited by the biological and physical tests known (Krieg, 1988). The bacterial genome on the other hand provides a source of material for bacterial identification that is independent of phenotypic characterizations (Krieg, 1988). DNA records the changes brought about by evolution, and the amount of difference between DNA sequences of different bacteria allow estimations in the divergence of those species. For example, Johnson (1973) used DNA/DNA homology experiments to show that previously phenotypically classified sub-species of *Bacteroides fragilis* were actually separate species of the *Bacteroides* genus, and have since been reclassified (Cato and Johnson, 1976). Because genotypic change occurs more rapidly than change in phenotypic characteristics, classifications based on genotype are more comprehensive and allow a greater discrimination between closely related organisms. Also, the recording of genotypic data is relatively simple as there are four defined nucleotides, as opposed to the assessment of physical tests (Olsen and Woese, 1993), which are fraught with ambiguities and open to interpretation.

Mutational changes in DNA can create and destroy restriction endonuclease sites and alter restriction fragment sizes by insertions or deletions. Restriction fragment length polymorphism (RFLP) is the process of analysing these differences. DNA is digested with restriction endonuclease which, when separated by gel electrophoresis, create patterns of different sized DNA fragments. Depending on the DNA tested and the type and distribution of restriction sites present, the pattern of restriction fragments may be unique for each bacterial species. Examining the patterns of restriction digests on a gel is complex as changes in the size of fragments may be small. However, hybridizing a nucleic acid probe to the digested, denatured DNA, creates simpler patterns for each bacterial species, depending upon the probe used for visualization (Grimont and Grimont, 1986). Probes to conserved regions of DNA (such as a region within the 16S ribosomal DNA, or species specific probes for determining differences between strains of one particular species (Flint *et al.,* 1990)) give distinct restriction fragment length polymorphisms. This allows differentiation of bacteria down to the strain level and can eventually allow characterisation of bacteria within a data base (Grimont and Grimont 1991).

Randomly amplified polymorphic DNA (RAPD) analysis also detects differences in DNA sequences (Williams et al., 1990). Short oligonucleotide primers (8-10 bases) are used to amplify DNA, using the polymerase chain reaction, from the organism of interest. The pattern of bands that result depends on the primers used. Some primers produce banding patterns that are unique to a particular organism. A number of primers may need to be assayed before one gives a pattern that is distinct for the bacterium in question. This technique has been successfully applied for the identification of *Helicobacter pylori* (Akopynaz et al., 1992), strain identification of *Listeria monocytogenes* (Czajka et at., 1992), *Xanthomonas campestris* pv pelargonii (Manulis et al., 1994), and in the identification of *Campylobacter* isolates (Mazurier et al., 1992).

An alternative application of RAPD analysis uses primers of longer length, but decreases the specificity with which they bind to the DNA for a limited number of cycles. The annealing stringency is then increased and this can produce unique patterns of DNA fragments (Welsh and McClelland, 1990). This technique has been applied by using t-RNA sequence as a basis for primer design (Welsh and McClelland, 1990). As

the t-RNA sequences are conserved, primers based on these have the potential to be utilized in all organisms (Welsh and McClelland, 1991). Both RAPD and RFLP techniques rely on the use of DNA extracted from organisms isolated from the environment and cultured *in vitro*. They do not provide any information on the classification of the organism, or any quantitative indication of presence of a bacterium in an environmental sample.

Another molecular method for bacterial identification is DNA/DNA reassociation. DNA/DNA reassociation relies on the ability of DNA from an organism to hybridize to complementary, labeled, single stranded DNA of similar sequence. The labeling allows quantitation of the reassociation between the DNA and hence an estimation of the similarity between the two sequences. Reassociation of 70% or greater is indicative of related bacterial species (Wayne *et al.,* 1987). As the data generated from these DNA homology experiments provides an evolutionary picture of the entire genome they can be used as an accurate method of determining bacterial phylogenetic relationships (Stackebrandt and Woese, 1981). The drawbacks of DNA/DNA reassociation are the length of time involved in these experiments and its limitations in determining long distance phylogenetic relationships (Krieg, 1988).

Using a single representative gene for phylogenetic analysis has the advantage of being a quick and simple method for data collection and analysis. However comparisons between most genes gives only a phylogenetic relationship for that gene, which isn't always representative of the phylogeny of the organism containing that gene (Stackebrandt and Woese, 1981). The translation apparatus (the ribosome and tRNA) are universally conserved molecules, prevalent in all organisms. These molecules are very similar in architecture across the three kingdoms and probably arose from a common ancestor. Phlyogenetic analysis of these molecules or parts of these molecules should allow relationships between organisms to be traced back to the common ancestor (Fox *et al.*, 1980). The rRNAs are key elements of the ribosome and are essential for protein synthesis. These are ancient molecules, extremely conserved in overall structure and this conservation in structure extends to conservation in the nucleic acid sequence. Some regions within the rRNA molecules do not vary between kingdoms, while other regions vary to a greater or lesser extent. The conserved sequences allow sequences to be aligned for phylogenetic analysis. The sequences of the rRNA molecules are long

enough to provide significant statistical information, and the rRNA genes appear to be free from lateral gene transfer. These features suggest that the rRNAs are very suitable for establishing phylogenetic relationships between organisms (Pace *et al.*, 1985).

In bacteria there are three ribosomal RNAs; the 5S, 16S and 23S subunits. All these subunits have been examined to varying degrees for the purpose of gathering bacterial phylogenetic information. Conservation in the structure of the 16S rRNA gene was first noted by Woese *et al* (1975), and 16S rRNA sequence was used as a basis for the structuring of the prokaryote domain into archaebacteria and eubacteria (Woese *et al.*, 1977). Application of the rRNA genes for environmental population analysis was first carried out by Stahl *et al* (1985) where 5S rRNA fragments were used to identify bacterial inhabitants of the Octopus thermal pool in Yellowstone National Park. However the information available in the 5S rRNA (ca. 250 nucleotides) is not sufficient to discriminate between closely related organisms. 16S rRNA is a larger molecule (ca. 1500 nucleotides) and contains enough information to give meaningful phylogenetic analysis, yet is small enough for practical handling (Pace *et al.*, 1985; Pace *et al.,* 1986). The larger size of the 23S rRNAs and its greater rate of change (Olsen and Woese, 1993) means they have not been used to the same extent as 16S rRNA for phylogenetic analysis.

The 16S rRNA genes contain both highly conserved regions and highly variable regions. Regions of high variability allow identification of relationships between closely related bacteria (Stackebrandt and Woese, 1981), while conserved regions (Woese *et al.,* 1975), determine relationships between distantly related bacteria. The most important feature of the 16S rRNA is that the phylogeny of rRNA genes is apparently representative of the phylogeny of the organism (Fox *et al.,* 1980). However, the varying rate of change within the 16S molecule means that the regions of rRNA being compared for estimates of homology are small, and less significant than entire genomes (Olsen and Woese, 1993). Therefore 16S rRNA sequence analysis gives broader outlines of the relationships between bacteria and is reliable for quick phylogenetic identification of bacterial species. However the examination of rRNA sequences will not replace the more sensitive DNA/DNA reassociation experiments which give more detailed information at lower taxonomic levels (Stackebrandt and Goebel, 1994).

Despite its drawbacks, 16S rRNA analysis has gained widespread acceptance as a means of phylogenetic analysis and has aided the classification of many bacterial species (Fox *et al.*, 1980). Initial phylogenetic relationships were determined by RNAase T1 oligonucleotide cataloguing, where 16SrRNA was digested with T1 ribonuclease, and the resulting fragments run on a two dimensional gel and sequenced, producing a catalogue of sequences characteristic for an organism (Fox *et al.*, 1980), but not a contiguous rDNA sequence. However advances in technologies have improved the speed and accuracy of 16S rRNA sequence determination. Sequencing of entire rRNA was performed using reverse transcriptase and oligonucleotide primers designed to the conserved regions of the 16S gene (Lane *et al.*, 1985). The polymerase chain reaction (PCR) eliminated the use of reverse transcriptase for sequencing as DNA rather than RNA can be sequenced with conserved primers applied directly to purified DNA and the PCR product directly sequenced (Edwards, *et al.*, 1989; Bottger, 1989; Weisburg *et al.*, 1990). This has led to the rapid compilement of a large data base of 16S sequences and over 2000 16S rRNA sequences are currently deposited in the Ribosomal Database Project (Olsen *et al.*, 1993).

1.3.2 Phylogenetic analysis of rumen bacteria.

Recently, molecular biology techniques have been applied to the classification of rumen organisms. RFLPs, DNA/DNA hybridisation and 16S rRNA analysis have been used as tools for further identification and reclassification of rumen bacteria.. The reclassification of *P. ruminicola* into four separate species has been proposed on the basis of DNA/DNA hybridisation, RFLP's, 16S rDNA sequencing, G+C content, signature oligonucleotide amplification and total cell protein profiles (Avgustin *et al.*, 1994; Avgustin *et al.*, 1997), and *B. fibrisolvens* strains have been shown to fall into two distinct phylogenetic clusters by DNA/DNA hybridization (Mannarelli, 1988) and 16S rDNA analysis (Forster *et al.*, 1996; Willems *et al.*, 1996) and should probably be reclassified into several new genera and species. A high level of genetic diversity of *Fibrobacter succinogenes* strains previously unobserved with biochemical tests and phenotypic characterization was recognized through RFLP analysis (Flint *et al.*, 1990), and this data was supported with DNA/DNA reassociation information. Paster *et al* (1993) used 16S rRNA sequence to further characterize the hyper-ammonia producing

bacteria as the taxonomic positions of these bacteria were unclear based on phenotypic testing. As a result of the investigation, two of the isolates were found to be closely related to existing species, *P. anaerobius* and *C. sticklandii.* The third bacterium was not closely related to any bacteria on the basis of 16S rDNA sequence and was classified as a new species, *C. aminophilum.*

The above examples demonstrate the utility of molecular techniques for classifying new isolates and reclassifying existing strains into coherent groups. However these techniques are only useful for classifying, and not enumerating bacteria. Oligonucleotide probing has proved to be a specific and successful method for determining bacterial populations in the rumen. Oligonucleotides are developed specific for a region of DNA in the bacteria of interest and are tested against other bacteria for specificity *in vitro* before the probes are applied to environmental samples. Different methods have been applied for the design of the oligonucleotide probes.

In a study of the proteolytic bacterium *Prevotella ruminicola* subsp. *brevis* $B_1 4$, Attwood *et al.* (1988) generated a recombinant genomic library of the organism and the resulting clones were tested for specificity against other *Prevotella* strains, *Selenomonas ruminantium* and *E. coli* using dot blot analysis. One clone carried DNA that proved to be specific for *P. ruminicola* subsp. *brevis* and this was used to follow the fate of this organism when introduced into the rumen. However the probe could not detect the *Prevotella* strain below a sensitivity limit of 2×10^7 bacteria (or 50 ng bacterial DNA). The poor sensitivity of this technique was probably due to the low or single copy number of the cloned gene sequence within the *P. ruminicola* genome (Brooker *et al.*, 1990). This method of bacterial detection is very labour intensive, and requires screening of multiple clones for specificity.

The 16S rRNA gene was first used as a target for species specific probes by Stahl and coworkers (1988). The variable regions within the 16S gene (Neefs *et al.*, 1990) were used to design probes specific for both a rumen and cecal strain of *Fibrobacter succinogenes* individually as well as a more general *F. succinogenes* probe. A probe specific for *Lachnospira multiparus* was also designed. These were applied to follow bacterial populations of *F. succinogenes* and *L. multiparus* before, during and after addition of the antibiotic monensin to the rumen. Expression of the bacterial

populations was reported as a proportion of the total 16S like rRNA population, determined by dot-blot analysis where universal probes were used to estimate the amount of total rRNA, and the intensity of the dot blot of the specific type probes compared to the total rRNA blots. However, use of rRNA as a method of quantitation is only an approximate estimate of bacterial numbers. The amount of rRNA in the cell changes during cell growth, making quantitation of bacterial numbers difficult to assess.

The 16S rRNA gene has been used for determining bacterial populations in the rumen or chemostat for a variety of bacteria. Briesacher *et al* (1992) used the probes designed by Stahl *et al* (1988) to successfully follow the population of *F. succinogenes* after feeding and supplementation with protein. Populations of *Synergistes joneseii* (McSweeny *et al*, 1993), *Ruminicoccus albus, R. flavefaciens* and *F. succinogenes* (Odenyo *et al*, 1994a, 1994b) *C. aminophilum, C. sticklandii* and *P. anaerobius* (Krause and Russell, 1996) have also been successfully followed *in vitro*.

The studies outlined illustrate that it is possible to identify specific populations of bacteria in the rumen by hybridizing probes to cellular rRNA or rDNA. Enumeration of these bacteria has previously been achieved by expressing the presence of specific rRNA as a proportion of total rRNA. However this gives only an approximate estimate of an individual organisms contribution to the total population as the amount of rRNA in a cell at any given time depends on the growth stage of that cell, and these results probably reflect the organisms contribution to metabolic activity rather than the contribution to population density (Stahl *et al*, 1988). Also, it has been recently been demonstrated that domain specific variations in dissociation temperatures occur with all universal probes previously used for determination of microbial populations (Zheng *et al*., 1996). The stability of target-probe duplexes formed between the universal probe and target DNA varies depending on what bacterial domain the target belongs to. A probe that binds to all domains with equal stability is desirable, but probes with a high level of variation in stability, could lead to significant bias when determining environmental microbial populations. *F. succinogenes* and *L. multiparus* populations were determined from rumen samples (Stahl *et al*., 1988; Briesacher *et al*., 1992; May *et al*., 1993) by normalising the population using a universal probe which has now been shown to have a high level of variation in binding to the different bacterial domains (Zheng *et al*., 1996), casting doubt on the results reported by these studies.

Another limiting factor in the use of oligonucleotide probes is the sensitivity of detection, which is governed mainly by the amount of rRNA present in the cell. As this is correlated to the growth rate those cells which are growing at a faster rate and contain more rRNA will be more easily detected. The detection limit for unique 16S rRNA probes in the rumen is 0.005% of 16S-like rRNAs, but for rRNA isolated from rumen contents this is higher at 0.01%, due to the plant rRNA (Stahl *et al.*, 1988). However these techniques are not sensitive enough to detect small populations of bacteria. A technique is therefore required which can determine populations of bacteria at low concentrations and express the population in terms of real numbers.

1.3.3 Competitive PCR

The polymerase chain reaction (PCR) is a powerful tool allowing amplification of regions of DNA using a thermostable polymerase (Mullis and Faloona, 1987; Saiki, *et at.*, 1988). It has had widespread application in food, medical and environmental microbiology for detection of bacteria (Bej *et al.*, 1991; Bej and Mahbubani, 1991), and is sensitive enough to detect a single bacterium in the environment (van Kuppeveld *et al.*, 1992). However quantitation using PCR is difficult due to the exponential manner in which the DNA amplifies, as variations between sample preparation and reaction conditions can not be excluded, and minor differences are magnified during the amplification process making comparison between samples less than satisfactory. The addition of an internal standard to PCR reactions, accounts for the variation between reactions, and allows accurate quantitation of DNA by competitive PCR (cPCR). In cPCR target DNA and internal control DNA compete for primers, reagents and enzyme in a competitive manner, producing products which are distinguishable from each other in an agarose gel. Both the control DNA and the target DNA are subject to identical reaction conditions and as the amount of amplified product is proportional to amount of starting product, the amount of starting product can be described by the formula log (N_{n1}/N_{n2}) = log $N_{01}/N_{02})$ + n log (eff_1/eff_2) (Zachar *et al.*, 1993). Amplification efficiencies must be equal to ensure accurate quantitation (Raemakers, 1993). Using this equation, unknown starting amounts can be determined by co-amplification of a known amount of internal control with the unknown DNA. The resulting log ratios of intensities are then compared to standard curves prepared from known target DNA and internal control.

This technique has been used to undertake quantitation of bacteria from environmental samples (Leser *et al.,* 1995; Lee *et al.,* 1996). The population of genetically engineered *Pseudomonas* sp. strain B13 (FR1) was followed after introduction into a laboratory microcosm by cPCR (Leser *et al.,* 1995). An internal control of 512 bp which co amplified with a fragment of 712 bp from the *Pseudomonas* sp. strain B13 (FR1) genome, was constructed by deleting a fragment from the 712 bp fragment (Leser,1995). Standard curves relating the intensity of the target PCR product to the intensity of the internal control were prepared, and used to quantitate the population of *Pseudomonas* sp. strain B13 (FR1) after introduction into the marine environment (Leser *et al.,* 1995). Results obtained with cPCR and colony counting did not agree (Leser *et al.,* 1995), possibly because of the detection of dead cells by PCR, or because that the cells entered a starvation-survival state, thus not reviving on selective media within the specified time frame. However, increases in population which were observed by cPCR which were not detected by specific culturing demonstrate that cPCR is a more sensitive method of detection than traditional culturing techniques

A more diverse use of cPCR is to enumerate bacteria which have not previously been cultured. Shot-gun cloning of 16S rDNA sequences from environmental samples can result in the identification of bacteria which have not previously been isolated. However because of the inability to study these bacteria on artificial substrates, population estimates can not be obtained. PCR primers unique to the uncultured organism can be designed by aligning the 16S rRNA sequence with the extensive 16S database available. This approach was used by Lee *et al* (1996), who shot-gunned cloned bacteria from a soil sample. The internal control was constructed by insertion of a 121bp fragment within the cloned 16S gene of the uncultured organism. Co-amplification was carried out on soil samples and compared to the standard curves previously constructed. This method gave an indication of the number of 16S rRNA gene copies present in the soil sample. However this can not be related to the number of bacteria present in the soil sample as the copy number of rRNA genes is unknown, but estimates can be drawn from likely copy number.

Competitive PCR has proven to be a sensitive method for quantitation of bacteria in the environment. Combined with the specificity of a primer designed to the 16S rRNA

gene of the organism of interest, then PCR should prove a sensitive, specific and quantitative assay for enumeration of bacteria in the rumen.

1.4 AIMS AND OBJECTIVES

Most of the information available on rumen nitrogen metabolism comes from studies done in the Northern Hemisphere on animals grazing dried forage and concentrate diets. However there is little corresponding information from animals grazing fresh forage under New Zealand conditions. The limited information obtained (Attwood and Reilly, 1995) has shown a difference in the proteolytic bacterial populations between the New Zealand animals and their Northern hemisphere counterparts, based on culture in non-selective media. The major contributors to rumen proteolysis identified under New Zealand conditions need to be investigated further to positively identify them and to determine numbers *in vivo*.

The identification of rumen bacteria is difficult and there are several examples of rumen bacteria being misidentified and then being reassigned to new species or genera (Flint *et al.*, 1990; Mannarelli *et al.*, 1990; Shah and Collins, 1990; Mannarelli *et al.*, 1991). Analysing bacterial nucleic acids provides a comprehensive and reliable method for bacterial identification. As pointed out previously, the 16S ribosomal RNA gene provides a compact source of genetic information, which allows rapid determination of bacterial relationships. It also contains sites of unique sequence which can be utilised for probe design. Moreover, 16S rRNA probes have been used to follow populations of rumen bacteria *in vitro* and *in vivo* (Stahl *et al.*, 1988; Briesacher *et al.*, 1992; May *et al.*, 1993; McSweeney *et al.*, 1993; Odenyo *et al.*, 1994a; Odenyo *et al.*, 1994b). However this method isn't quantitative, and small populations of bacteria may be missed. Also, quantitation of bacteria using this method is expressed only in relative amounts to total RNA present, and gives no direct indication of numbers of bacteria present.

The sensitivity limitations of the 16S rRNA probes may be over come by adapting the method for use with the polymerase chain reaction. The specificity of the 16S rRNA probes can be retained by using these oligonucleotide sequences to prime a polymerase chain reaction. As PCR has the potential to amplify one molecule of DNA 10^6 times (Saiki *et al.,* 1988), a large increase in probe sensitivity is possible. Furthermore, PCR has been used to successfully follow populations of bacteria in the environment in a quantitative manner (Bej et al., 1991; Bej and Mahbubani, 1992).

The aim of this study is to determine the 16S rRNA sequences of prominent proteolytic rumen bacteria from New Zealand ruminants, and to use this to classify these bacteria. Also, the 16S rRNA sequences will be used to design specific oligonucleotides which will be tested as primers in PCR reactions to enumerate the major proteolytic rumen bacteria using a competitive PCR technique.

Chapter 2 - METHODS AND MATERIALS

2.1 BACTERIAL STRAINS

The bacterial strains used during this work are listed in Table 2.1.

Table 2.1. Bacterial strains

Bacterium	Strain	Source
Butyrivibrio fibrisolvens	C130a, C211, C219	Attwood and Reilly,1995
Butyrivibrio fibrisolvens	CF3, H17c	M. P. Bryant, University of Illinois
Butyrivibrio fibrisolvens	WV1	lab strain
Butyrivibrio fibrisolvens	OR509	R. Teather, Centre for food and Animal Research, Agriculture and Agri-food, Canada
Butyrivibrio fibrisolvens like	C122a, C21b	Attwood and Reilly,1995
Clostridium aminophilum	F	J.B. Russell, Cornell University
Clostridium clostridiforme	ATCC 25537, ATCC 29084	ATCC
Clostridium proteoclasticum	ATCC 51982	Attwood and Reilly, 1995
Clostridium sticklandii	SR	J.B. Russell, Cornell University
Enterococcus faecalis	NCTC 775	
Enterococcus faecalis	68a	lab strain
Escherichia coli	DH5-α	Gibco BRL
Eubacterium cellulosolvens	5494	M. P. Bryant, University of Illinois
Eubacterium limosum	ATCC 8486	〃
Eubacterium ruminantium		〃
Eubacterium sp.	C11, C12b, C13b, C14b#2, C118b, C119b, C120a, C124b, C125b, C260,	Attwood and Reilly,1995
Fibrobacter succinogenes	AC3	M. P. Bryant, University of Illinois
Lachnospira multiparus	ATCC 19307	〃
Megasphaera elsdenii	T81	〃
Peptostreptococcus anaerobius	C	J.B. Russell, Cornell University
Peptostreptococcus productus	SF-50	M. P. Bryant, University of Illinois
Prevotella ruminicola	C21a	Attwood and Reilly,1995
Prevotella ruminicola brevis	118b, B14	M. P. Bryant, University of Illinois
Prevotella ruminicola ruminicola	23	〃
Ruminococcus albus	7, 8	〃
Ruminococcus flavefaciens	ATCC 19208, fD1*	〃
Selenomonas ruminantium lactilytica	GA31	〃
Selenomonas ruminantium ruminantium	ATCC 12561, ATCC 27209	〃
Streptococcus bovis	JB1	〃
Streptococcus bovis	NCFB 2476	
Streptococcus bovis	3-31, 3-36, 3-39, 5-21, 7-2, 7-25, 7-26.	J.B. Russell, Cornell University

Bacterium	Strain	Source
Streptococcus bovis	A12, A14, A120, A166, A191b, B11, B32a, B314, B315, B327, B337, B342, B346, B348, B350, B360, B372, B382, B385, B395, B396, B398, C14b#1, C17, C123b, C271.	Attwood and Reilly,1995
Streptococcus bovis	RF-1, RF-2, UDY-KF	lab strains
Succinomonas amylolytica	ATCC 19206	M. P. Bryant, University of Illinois
Succinovibrio dextrinosolvens	ATCC 27209, ATCC 19716	"

2.2 CHEMICALS

Chemicals used for media preparation were reagent grade. Those used for buffers and reagents for molecular biology were Analar grade or higher. Suppliers of special chemicals are indicated in the appropriate section describing the use of those chemicals.

2.3 MEDIA

All media used for bacterial growth are listed in Appendix A

2.4 BUFFERS AND SOLUTIONS

All buffers and solutions used are listed in Appendix B

2.5 BACTERIAL GROWTH

A variation of CC Medium (Leedle and Hespell, 1980) in which glucose (10 g l^{-1}) replaced all carbohydrates (CC-Glu) was used for the growth and maintenance of all anaerobic bacterial strains. *Ruminococcus albus* and *R. flavifaciens* were grown on CC medium in which cellobiose (10 g l^{-1}) was supplied as the carbohydrate source, while *Clostridium aminophilum, C. sticklandii* and *Peptostreptococcus anaerobius* were grown on CC medium in which no carbon source was added and tryptone (Difco, (Detroit, USA) 1.5% wt/vol) replaced trypticase. Cultures were grown under strictly anaerobic conditions at 39°C.

E. coli broth cultures were incubated aerobically with vigorous shaking at 38°C.

2.6 PHENOL/CHLOROFORM/ISOAMYL ALCOHOL EXTRACTIONS

Buffer saturated phenol (pH 8.0, see Appendix B), chloroform and isoamyl alcohol were combined in a 25:24:1 (v/v/v) ratio, and an equal volume of this mixture was added to the solution to be extracted. The solution was mixed vigorously using a cyclo mixer, centrifuged at 10 000 x g for 5 minutes and the aqueous phase removed to a fresh tube. Extractions were repeated until the organic/aqueous interface was free of protein. The aqueous phase was finally extracted with an equal volume of chloroform/isoamyl alcohol (24:1).

2.7 ETHANOL PRECIPITATIONS

DNA in solution was precipitated by adding one tenth volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol and incubation at -20°C for at least 1 hr. Precipitated DNA was recovered by centrifugation at 12 000 x g at 4°C for 20 min. The supernatant was removed and the pellet washed with 70% ethanol. After recentrifugation the supernatant was removed and the pellet was air dried.

2.8 DNA EXTRACTION

Three different methods of DNA extraction were used.

2.8.1 Enzymatic Lysis (Saito and Miura, 1963)

Bacterial cultures grown overnight were collected by centrifugation at 16 500 x g, at 4°C for 10 minutes. Cuture supernatant was removed and discarded, and the bacterial pellet was resuspended in 10 ml saline-EDTA. Lysozyme (Boehringer Mannheim, Germany) (final concentration, 1 mg ml^{-1}), and RNase A (20 µg ml^{-1}) were added and incubated at 37°C until lysis had occurred or 2.5 hours. SDS (1% w/v) and protease K (0.2 mg ml^{-1}) were added mixed and incubated at 65°C for 2.5 hours. The lysate was phenol/Chloroform/Isoamyl alcohol extracted and the DNA was precipitated with ethanol.

2.8.2 Physical Disruption

To eliminate possible bias introduced by enzymatic cell wall lysis and to maximize DNA extraction efficiencies, physical disruption was used to extract DNA from rumen samples and from cells for sensitivity experiments and standard curve construction.

Unless otherwise stated, DNA was extracted from triplicate samples. One ml of homogenized rumen contents or 1 x 10^{10} bacterial cells were added to 1.2 g of sterile zirconia/silica beads (Biospec Products Ltd., OK., USA) followed by centrifugation at 12 000 g for 10 min at room temperature. The pellet and beads were rinsed twice in saline-EDTA solution, before final resuspension in 750 µl of saline-EDTA. Physical disruption was performed using a Mini-beadbeater (Biospec Products Ltd., OK., USA) at maximum speed for 2 intervals of 2 min, with a 1 min. incubation on ice between each treatment. The mixture was phenol/chloroform/isoamyl alcohol extracted to remove proteins and the nucleic acids were precipitated with ethanol and centrifuged at 10 000 g for 20 min at 4°C. The air dried pellet was resuspended in TE buffer and RNase A (1mg ml^{-1}) added and incubated at 37°C for 60 min. RNase A was removed by phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation and centrifugation. The air dried DNA pellet was resuspended in TE buffer and was stored at -20°C, until required.

2.8.3 Chemical Extraction

DNAzol reagent (Gibco BRL, Life Technologies, Auckland, New Zealand) was used for chemical extraction of DNA. Cells were suspended in 1ml of DNAzol reagent before bead-beating as above. The DNA was then precipitated according to manufacturer's instructions, by adding 500ul of absolute ethanol, and inverting the tube to mix contents. The resulting DNA was pelleted by centrifugation at 2000 x g for 2 minutes at 4°C. The supernatant was removed and the pellet washed twice in 1 ml of 95% ethanol. The pellet was air dried, and resuspended in TE.

2.9 DNA Quantitation

DNA concentration and purity were determined spectrophotometrically measuring $A_{260/280}$ using a Spectramax microplate spectrophotometer (Molecular Dynamics, CA., USA).

2.10 Agarose gel electrophoresis

Depending on the size of the DNA, samples were analyzed on 0.8, 1.2 or 2 % (w/v) MP agarose (Boehringer Mannheim, Germany) in 1 x TAE buffer. Where DNA was to be recovered from agarose, 1.5 or 2.5% Low melting point agarose (Sigma Chemical Co., Mo, USA.) was used. DNA samples were mixed with 6 x loading buffer, loaded into

the wells and electrophoresed at 8 - 12 V cm^{-1} for 1 - 2 hours. After electrophoresis, gels were stained with a 5 μg ml^{-1} solution of ethidium bromide for 10 minutes and destained for 20 min in water. Agarose gels were viewed on a UV-transilluminator, and photographed using Polaroid 667 film with a Polaroid MP-4 land camera (St. Albans, Hertfordshire, England).

2.11 Extraction of DNA from Agarose

A modification of the freeze-squeeze technique was used. The DNA band of interest was excised from a low melting point agarose gel and melted at 65°C. An equal volume of buffer equilibrated phenol was added, mixed and immediately frozen at -20°C and left overnight. The phenol/agarose mixture was centrifuged at 10 000g for 10 min at room temperature, and the aqueous phase removed and re-extracted with phenol/chloroform/isoamyl alcohol. The aqueous phase was then ethanol precipitated and the resulting DNA resuspended in TE or distilled sterile water.

2.12 Restriction digests

Restriction digests were carried out in 20μl reaction volumes in 1.5 ml microfuge tubes, using buffers supplied by the manufacturer. Reactions contained 0.5-1.0 μg of DNA, 2μl of supplied 10x buffer and 5-10 units of restriction enzyme. Incubations were carried out at the recommended temperature for at least 3 hours.

2.13 Ligations

Ligations were carried out in 20 μl reaction volumes in 1.5 ml microfuge tubes using the 5x reaction buffer supplied by the manufacturer. Reaction contained DNA at a vector:insert ratio of approximately 1:3, 4 μl of 5 x reaction buffer and 10 units of T4 DNA ligase (Gibco BRL, Life Technologies, Auckland, NZ) and were incubated at 23°C for 1.5 hours to facilitate the ligation of the of cohesive termini. Ligation reactions were diluted to 100 μl before use in transformations or PCR reactions.

2.14 TRANSFORMATIONS

A modified protocol of Sambrook *et al* (1989) was used for the transformation of plasmid DNA into *E. coli* DH5-α competent cells (Gibco BRL, Life Technologies, Auckland, New Zealand). Approximately 10ng of transforming DNA was added to 100µl of competent DH5-α *E. coli* cells. The contents of the tubes were mixed gently and allowed to sit on ice for 30 min. Cells were heat shocked at 42°C for 45 sec, and quickly chilled on ice for 1-2 min. Cells were diluted with 800µl of SOC medium and incubated at 37°C with gentle shaking for 45 min, before aliquots of transformed cells were plated onto SOB solid medium, containing, ampicillin (50 µg ml^{-1}), isopropyl-β-D-thiogalactopyranoside (0.16 mg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (50 µg ml^{-1}) Plates were incubated overnight at 38°C.

2.15 PLASMID DNA MINIPREPARATIONS

Plasmid DNA was recovered from *E. coli* cells using the alkaline lysis method described by Sambrook *et al.*, (1989). Composition of buffers used are listed in Appendix B. Five ml of LB medium containing ampicillin (50 µg ml^{-1}) was inoculated with a single colony and incubated overnight with vigorous shaking. 1.5 ml of culture was transferred aseptically to a microfuge tube and was centrifuged in a bench top centrifuge for 2 min. The supernatant was removed and the bacterial pellet was resuspended in 100 µl TEG with vigorous vortexing. Two hundred µl of a freshly prepared 0.2 M NaOH/1% SDS solution was added, and the tube rapidly inverted several times before storing on ice. 150µl of solution III (final concentration, 3M Na and 5M Acetate) was added and the tube inverted again to mix contents, and stored on ice for 3-5 min. The microfuge tube was centrifuged at 12 000 g at 4°C for 5 min and the supernatant carefully collected, and extracted once with phenol/chloroform/isoamyl alcohol. The aqueous phase was ethanol precipitated and rinsed in 70% ethanol and finally resuspended in 50 µl of TE until further analysis.

2.16 PCR PRIMERS AND AMPLIFICATION

The primers used for PCR reactions are listed in Table 2.2.

Table 2.2. PCR primers

PCR Primer	Description	Sequence (5'-3')
fD1*	16S universal forward	GAGTTTGATCCTGGCTCAG
rD1*	16S universal reverse	TAAGGAGGTGATCCAGCC
B315 454	*S. bovis* specific	CTTTCCACTCTCACACACG
B316 830	*C. proteoclasticum* specific	CTGAATGCCTATGGCACCCAA
fD1	16S universal forward. + linker	CCGAATTCGTCGACAACAGAGTTTGA TCCTGGCTCAG
rD1	16S universal reverse + linker	CCCGGGATCCAAGCTTAAGGAGGTG ATCCAGCC

Both the B316 830 and the B315 454 primers were screened for specificity using the PROBE CHECK program of the Ribosomal Database Project (Olsen *et al.*, 1992) and NCBI BLAST search. PCR amplification of *C. proteoclasticum* DNA produces an 830 base pair product when amplified with fD1* and B316 830, while *S. bovis* DNA produces a 473 bp product when amplified with fD1* and B315 454.

PCR reactions contained 20 mM Tris. HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 μM of each primer and 0.5 U of Taq DNA polymerase (Gibco BRL, Life Technologies, Auckland, New Zealand). PCR reactions were in a final volume of 20 μl sealed in a capillary tip and thermocycling was carried out in a Corbett FTS-1 Capillary Thermal Sequencer (Corbett Research, Sydney, Australia). PCR amplification conditions for B316 830/fD1*and B315 454/fD1* primer pairs were denaturation at 95°C for 3 min, followed by 6 cycles of 95°C for 30s, 62 °C for 15s, 72°C for 30s and 25 cycles of 95°C for 15s, 62°C for 5s and 72°C for 30s and a final cycle of 72°C for 3 min. Amplification with the universal primers, fD1*/rD1* or fD1/rD1differed only in annealing temperature, which was 55°C. PCR products were

separated by electrophoresis in agarose gels, stained with ethidium bromide and visualized by UV trans-illumination.

2.17 CLONING THE 16S rRNA GENES

The 16S rRNA genes of the proteolytic bacteria were cloned into plasmid vectors to facilitate DNA sequencing. The genomic DNA of the organism was amplified with the universal PCR primers fD1 and rD1 which specifically amplify the 16S rRNA gene of most bacteria. The primers were constructed with linkers at the 5' end containing restriction endonuclease sites. After amplification the DNA band corresponding to the 16S rRNA gene was extracted from the agarose and the DNA double digested with *Eco*RI and *Bam*HI (Boehringer Mannheim, Germany). Because of a conserved internal *Eco*RI site, the 1500 bp fragment was cloned in two pieces. The DNA fragments were ligated into pUC19 digested either with *Eco*RI alone or *Bam*HI and *Eco*RI (Fig. 2.1). The ligations were transformed into *E. coli* DH5-α cells, and white colonies were screened for inserts of the correct size. Plasmids were prepared for sequencing using Qiagen plasmid purification kits.

2.18 16S rDNA SEQUENCING

Plasmid DNA was prepared for sequencing using QIAGEN (Qiagen GmbH, Germany) plasmid kit. Reagents and buffers are listed in Appendix B. Three ml of LB broth containing ampicillin (50 $\mu g\ ml^{-1}$) was inoculated with plasmid containing *E. coli* clone, and incubated over night at 37°C. The culture was centrifuged at 10 000 x g for 2 min and DNA extracted from the pelleted bacteria following the manufacturer's instructions. Purified plasmid DNA was resuspended in water at 200ng ul^{-1}

DNA sequencing was performed by DNA Sequencing Facility (School of Biological Sciences, Auckland University) using dideoxychain terminator reaction on a ABI 373A automated sequencer (Applied Biosystems, Foster City, California). Both forward and reverse strands were sequenced. Primers were supplied at a concentration of 3.2 pmol μl^{-1}, for use in dye terminator reactions and are listed in Table 2.3.

Figure 2.1. Cloning of the 16S rRNA genes.

The 16S rRNA gene was amplified from genomic DNA with the universal primer fD1 and rD1. The PCR product was digested with *Eco*R1 and *Bam*H1 to give two fragments of approximately 670 and 830 bp in length. The fragments were ligated into the plasmid pUC19, previously digested with *Eco*R1 alone or with *Eco*R1 and *Bam*H1.

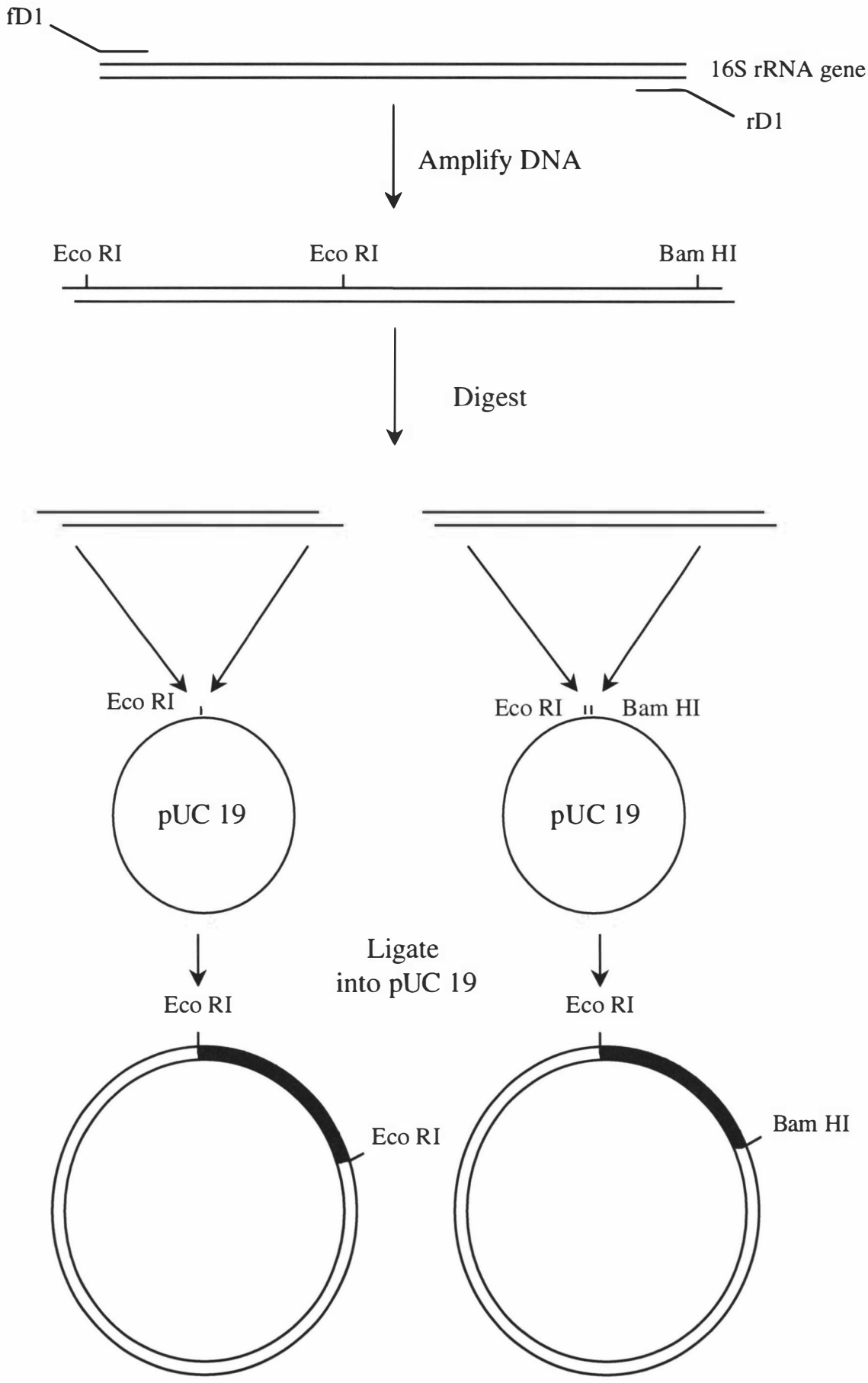

fD1
16S rRNA gene
rD1
Amplify DNA
Eco RI
Eco RI
Bam HI
Digest
Eco RI
Eco RI
Bam HI
pUC 19
pUC 19
Ligate
into pUC 19
Eco RI
Eco RI
Eco RI
Bam HI

Table 2.3. Sequencing Primers

Sequencing Primers	Sequence (5'-3')	Position (5'-3' *E. coli* numbering)
M13/pUC forward	CCCAGTCACGACGTTGTAAAACG	pUC19
M13/pUC reverse	AGCGGATAACAATTTCACACAGG	pUC19
forRNA F1	CGTGCCAGCAGCCGCGGTAA	513-532
forRNA F3	TCCCGCAACGAGCGCAACCC	1094-1113
RevRNA R5	GCTTGTGCGGGCCCCCG	939-923
revRNA R6	GGGTTGCGCTCGTTGCGGGA	1113-1096
fD1*	GAGTTTGATCCTGGCTCAG	8-28
F1	CTCCTACGGGAGGCAGCAG	339-357
F2	CAGGATTAGATACCCTGGTAG	785-805
R1	CTGCTGCCTCCCGTAA	357-342

The 16S rDNA sequences of *S.* bovis strain B315 and *B. fibrisolvens*-like strain B316 were confirmed by sequencing directly from PCR product. Genomic DNA was PCR amplified using the universal primers fD1* and rD1* and the 16S rDNA fragment was separated in 1.2% low melting point agarose gel. The band of correct size was excised from the gel, diluted in 100 µl of TE, and was homogenized to a fine slurry using a pipettor. The slurry was loaded into a Micropure™ (Amicon Inc., Ma., USA) spin column, and centrifuged to remove the solid agarose. The liquid which passed through the column was loaded into a Microcon®-100 (Amicon Inc., Ma., USA) sample reservoir and was spun for 14 min at 500 x g at room temperature. 450 µl of water was added to the column and spun for a further at 500 x g for 14 min. The DNA was recovered from the spin column by inverting into a fresh vial, and a further centrifugation at 500g for 2 min. The concentration of the DNA was determined by measurement of $A_{260/280}$ and the DNA was diluted to 50 ng μl^{-1} for sequencing directly.

2.19 SEQUENCE ANALYSIS

Sequence fragments were assembled using the McMolly DNA Handling Program for Macintosh computers and the SeqMan program of the DNA* package. Closely related sequences were identified using the BLAST function of the National Center for Biological Information and the Similarity-Rank program of the Ribosomal Database

Project (RDP). Sequences were retrieved from the Genbank and EMBL databases. The 16S rRNA sequences were aligned using Clustal X version 1.64b, and corrected manually. The phylogenetic trees were constructed using the Maximum Likelihood program contained in the Phylip package.

2.20 CONSTRUCTION OF THE INTERNAL CONTROLS

The 830 bp PCR product from *C. proteoclasticum* DNA amplified with fD1* and B316 830 contains two *Alu*I sites. To produce an *Alu*I deletion, the 830 bp fragment was digested with *Alu*I (Boehringer Mannheim, Germany) and the restriction endonuclease was removed with phenol treatment followed by ethanol precipitation. The *Alu*I fragments were ligated with T4 DNA ligase (Gibco BRL, Life Technologies, Auckland, New Zealand). Two µl of the ligation reaction was used in a PCR reaction using fD1* and B316 830 and the products separated by agarose gel electrophoresis. One of the PCR products was a 480 bp fragment expected from the deletion of the 350 bp internal *Alu*I fragment. The 480 bp fragment was purified from the gel, and used as an internal control for cPCR reactions. The concentration of the internal control was estimated by intensity of fluorescence of the internal control when compared to standards from photographs of the gel at approximately 100 ng ml^{-1}. However the DNA concentration was too low to obtain an accurate $A_{260/280}$ reading, so the internal control was expressed as a dilution of the concentrated mixture.

The internal control for *S. bovis* was prepared in a similar manner to that of *C. proteoclasticum.* Digests of the 473bp rDNA fragment from *S. bovis* with *Alu*I produced five fragments which were phenol chloroform extracted and ethanol precipitated before religation with T4 DNA ligase. The ligation reaction was then used in a PCR reaction and the bands separated in 2.5% low melting point agarose gel. Three ligation products resulted from amplification, and each was extracted from the agarose using the modified freeze squeeze technique. The 117 bp amplification product was used as the internal control for determining *S. bovis* populations in the rumen.

2.21 PREPARATION OF BACTERIAL CELLS FOR SENSITIVITY TESTING

C. proteoclasticum or *S. bovis* were grown overnight in 100 ml cultures and a sample counted by phase contrast microscopy using a WSI counting chamber (Weber Scientific International Ltd., Middlesex, England). Cells were pelleted by centrifugation and resuspended to a final concentration of 1 x 10^{10} cells ml^{-1} for DNA extractions. Ten fold serial dilutions of this DNA were amplified to determine the detection limit of the assay.

2.22 QUANTITATION OF PCR PRODUCTS

PCR products were quantitated by photographing agarose gels using Polaroid 665 film (St. Albans, Hertfordshire, England) which produces a negative image of the photograph. The negative was scanned using a GS-670 Imaging Densitometer (BioRad, Hercules, CA. USA), and analyzed using Molecular Analyst Software version 1.4 (BioRad, Hercules, CA. USA.). To correct for differences in fluorescence of ethidium bromide stained PCR fragments of different sizes (Piatak *et al.,* 1993), the intensity of the internal control was multiplied by the ratio 830/480 for *C. proteoclasticum* or 473/102 for *S. bovis.*

2.23 RUMEN SAMPLES

Eight fistulated, lactating, Fresian dairy cows were sampled at the Dairying Reasearch Corperation in Hamilton as part of a feeding trial, in conjunction with Dr. Vicki Carruthers. The animals were fed 4 different diets in rotation: high nitrogen; high nitrogen with carbohydrate; low nitrogen and low nitrogen with carbohydrate. Ryegrass pasture, containing less than 5% clover, was top dressed with 20 kg of nitrogen (as urea) per hectare for low nitrogen diets, or 90 kg of nitrogen per hectare for high nitrogen diets. Urea was applied 21-28 days before cutting, and nitrogen was 2.11% and 2.82% of dry matter for low and high nitrogen diets respectively. Carbohydrate was supplied as a 50:50 mixture of dextrose/cornflour on an energy basis, and was drenched at 9:00 am, 11:00 am, 4:00 pm and 6:00 pm supplying 10% of minimum energy intake. Cows were fed at 9:00 am and 4:00 pm and were maintained on each diet for 12 days before sampling. The order of feed rotation is indicated in Table 2.4.

Table 2.4. Rotation of Diet

Cow	Treatment			
	Sample Date (1996)			
	19th September	4th October	24th October	9th November
709	High N	High N + CHO	Low N + CHO	Low N
710	High N + CHO	High N	Low N	Low N + CHO
727	High N	Low N + CHO	Low N	High N + CHO
788	Low N	High N + CHO	High N	Low N + CHO
1758	Low N	Low N + CHO	High N + CHO	High N
8702	Low N + CHO	High N	High N + CHO	Low N
9754	Low N + CHO	Low N	High N	High N + CHO
9775	High N + CHO	Low N	Low N + CHO	High N

Rumen samples of approximately 250 ml were collected prior to the last 4:00 pm feed and were frozen immediately in liquid nitrogen and stored at -80°C until DNA extraction. Immediately before DNA extraction samples were thawed in a 37°C water bath and diluted with an equal weight of Mineral Salts (MS) buffer before homogenization in a Sorvall tissue homogenizer (Du Pont Co. DE. USA.).

Chapter 3 - 16S rDNA DETERMINATION, ANALYSIS, PRIMER DESIGN AND INTERNAL CONTROL CONSTRUCTION

3.1 INTRODUCTION

The 16S rRNA gene has been widely used in the identification and phylogenetic analysis of bacteria, both for previously cultured organisms and for 16S genes cloned directly from environmental samples (Pace *et al.,* 1986; Amann, 1995; Lee *et al.,* 1996). The 16S rRNA sequence contains enough information to be representative of the entire genome and phylogenetic inferences can be drawn from this information (Woese *et al.,* 1975; Fox *et al.*, 1980; Pace *et al.,* 1986; Olsen and Woese, 1993). The 16S rRNA gene also provides sites for probe design at different taxonomic levels, and has been used successfully for enumerating bacterial populations in the rumen (Stahl *et al.,* 1988; Briesacher, *et al.,* 1992; May *et al.,* 1993; Krause and Russell, 1996).

PCR is an extremely sensitive and specific method of detection. However because of the logarithmic amplification of PCR small differences between reactions can be amplified, making quantitation by PCR difficult. The addition of an internal control to PCR reactions normalises for reaction to reaction variation, which makes quantitation by PCR possible. Competitive PCR (cPCR) was first developed for the quantitation of HIV-1 3B LTR DNA (Zachar *et al.,* 1993), but more recently, its use has been extended to quantitation of bacteria in the environment (Leser *et al.,* 1995; Lee *et al.,* 1996; Kobayashi *et al.,* 1998).

B. fibrisolvens-like strain B316 and *S. bovis* strains B315 and C14b#1 are proteolytic rumen bacteria that have been isolated from New Zealand ruminants, and characterised by phenotypic typing (Attwood and Reilly, 1995; Attwood and Reilly, 1996). In this study, the 16S rRNA genes of these organisms and a *S. bovis* reference strain, NCFB 2476, were sequenced in order to further characterise these bacteria and to design specific nucleic acid probes. Internal controls were also developed to use in conjunction with the specific primers for the quantitation of these bacteria in the rumen.

3.2 RESULTS

3.2.1 Cloning and sequence analysis of 16S rRNA genes

The almost entire 16S rRNA genes of *C. proteoclasticum* strain B316^T and *S. bovis* strains B315, C14b#1 and NCFB 2476 were cloned and sequenced and are listed in Appendix C. *C. proteoclasticum* was found to be most similar to *B. fibrisolvens* strain NCDO 2345 at 99.5% similarity. Other NCDO *B. fibrisolvens* strains 2434, 2432, 2398 and 2222, were also very closely related (Fig. 3.1). *S. bovis* strains B315, C14b#1 have almost identical 16S rRNA genes (Fig. 3.2), and these form a tight cluster with the *S. bovis* type strain NCDO 597, *S. equinus* and *S. bovis* isolate NCFB 2476.

3.2.2 Primer design

Alignments of the *C. proteoclasticum* and *S. bovis* 16S rDNA sequences were made with closely related sequences obtained from the Ribosomal Database Project (RDP) and Genbank. Regions of unique sequence were identified for both *C. proteoclasticum* and *S. bovis* and a primer specific for each bacterium was designed (Fig. 3.3). The primers selected, B316 830 and B315 454, predicted PCR products of 830 bp and 454 bp for *C. proteoclasticum* and *S. bovis* respectively. Primer specificity was checked using the Probe Check facility of the RDP, and both primers were found to be specific. However, when the primer sequences were subjected to BLAST searches, they showed homology with other 16S rDNA sequences. The *C. proteoclasticum* primer was found within the 16S rDNA sequences of the four closely related *B. fibrisolvens* strains; NCDO 2435, NCDO 2434, NCDO 2222 and NCDO 2398, while the *S. bovis* primer was present in *S. rattus, S. mutans, S. milleri, S. intestinalis, S. equinus, S. caprinus, S. constellatus, S. alactolyticus* and *S. macedonicus* as well as all published *S. bovis* strains.

Figure 3.1. Phylogenetic analysis of *C. proteoclasticum*

Unrooted phylogenetic tree constructed using a maximum likelihood method, showing the position of *C. proteoclasticum* in relation to the closely related *B. fibrisolvens* strains within cluster XIVa of the *Clostridium* subphylum of the Gram positive bacteria. The bar represents a sequence divergence of 1%.

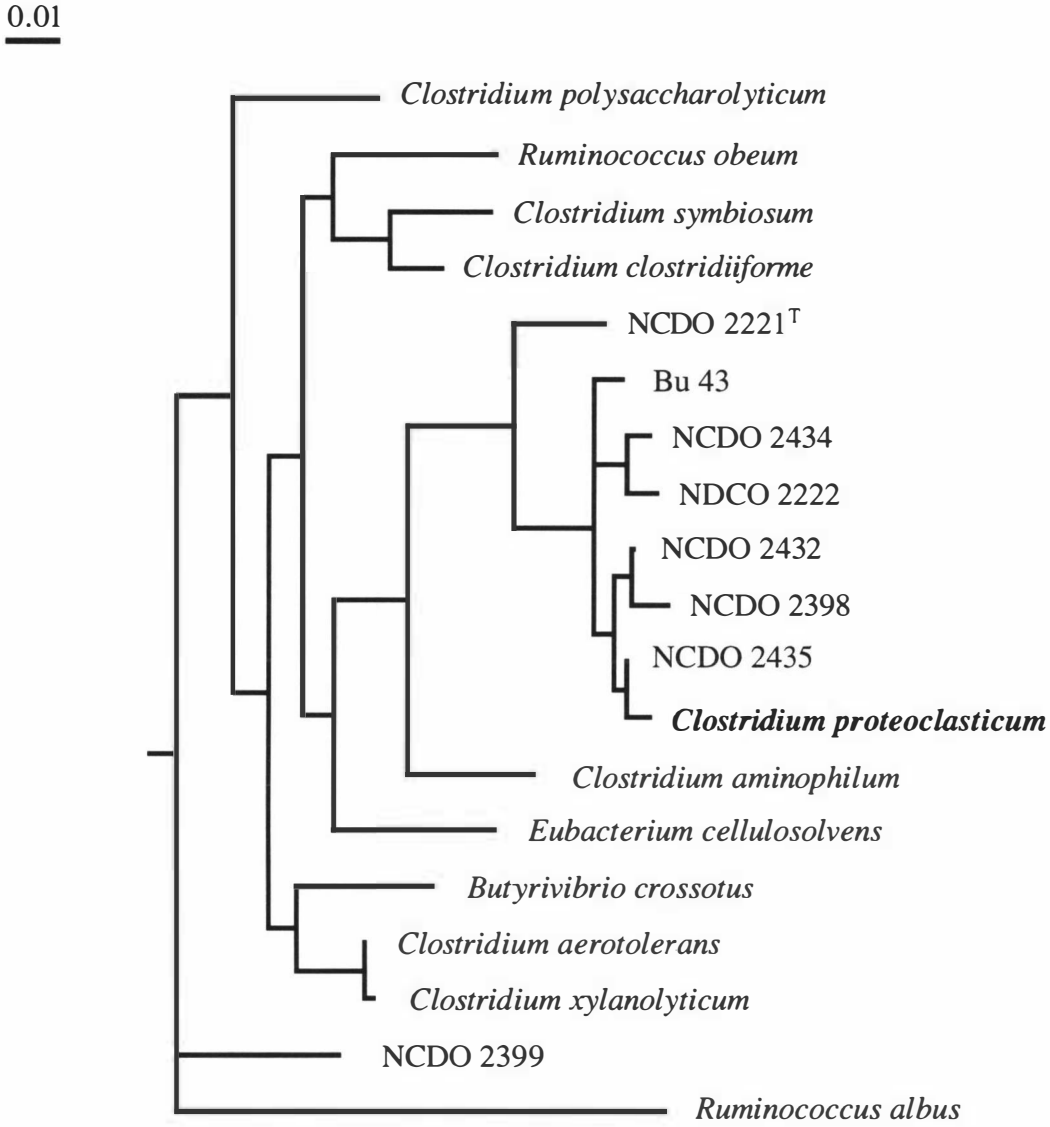

0.01
Clostridium polysaccharolyticum
Ruminococcus obeum
Clostridium symbiosum
Clostridium clostridiiforme
NCDO 2221T
Bu 43
NCDO 2434
NDCO 2222
NCDO 2432
NCDO 2398
NCDO 2435
Clostridium proteoclasticum
Clostridium aminophilum
Eubacterium cellulosolvens
Butyrivibrio crossotus
Clostridium aerotolerans
Clostridium xylanolyticum
NCDO 2399
Ruminococcus albus

Figure 3.2. Phylogenetic analysis of streptococcal strains.

Unrooted phylogenetic tree constructed using a maximum likelihood method, showing the relationships of the New Zealand streptococcal strains, B315 and C14b#1 and strain NCBF 2476 to other streptococcal species. The bar represents a sequence divergence of 1%.

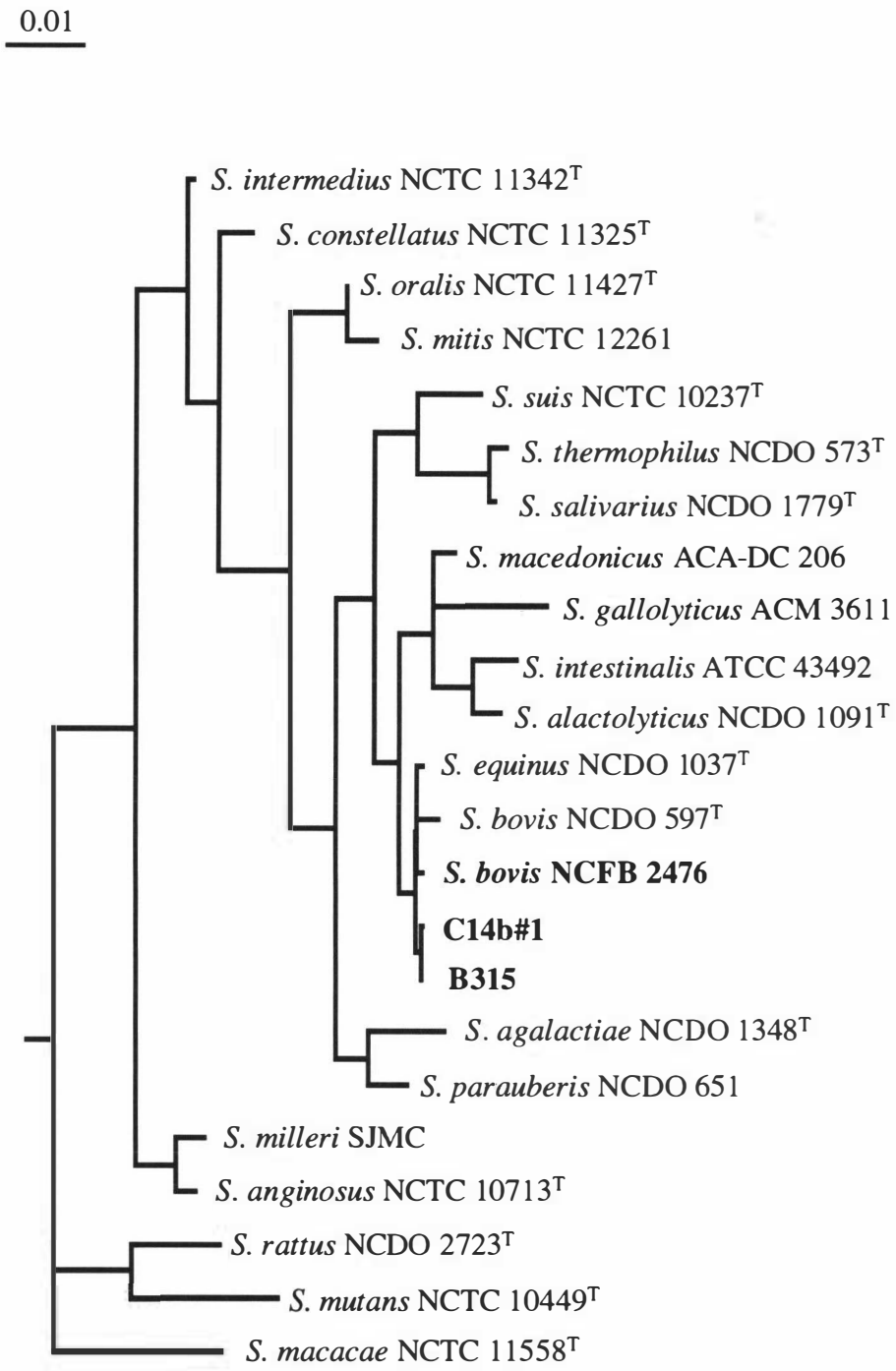

0.01
S. intermedius NCTC 11342T
S. constellatus NCTC 11325T
S. oralis NCTC 11427T
S. mitis NCTC 12261
S. suis NCTC 10237T
S. thermophilus NCDO 573T
S. salivarius NCDO 1779T
S. macedonicus ACA-DC 206
S. gallolyticus ACM 3611
S. intestinalis ATCC 43492
S. alactolyticus NCDO 1091T
S. equinus NCDO 1037T
S. bovis NCDO 597T
S. bovis NCFB 2476
C14b#1
B315
S. agalactiae NCDO 1348T
S. parauberis NCDO 651
S. milleri SJMC
S. anginosus NCTC 10713T
S. rattus NCDO 2723T
S. mutans NCTC 10449T
S. macacae NCTC 11558T

Figure 3.3. Primer design for *C. proteoclasticum* and *S. bovis.*

The 16S rRNA sequences of *C. proteoclasticum* and *S. bovis* were aligned with the 16S rRNA sequence from closely related bacteria and the variable regions identified.

a) The B316 830 primer begins at 832 bp (*E. coli* numbering) and is 21 bp in length. The primer circumscribes *C. proteoclasticum* and four closely related *B. fibrisolvens* strains.

b) The B315 454 primer begins at 454bp (*E. coli* numbering) and encompasses 11 streptococcal species.

a

	824								862
B316	CTAGGTG	TTG	GGTGCCAT.A	GGCATTCAG	TGCCGTCGCTA				
NCDO 2435	CTAGGTG	TTG	GGTGCCAT.A	GGCATTCAG	TGCCGTCGCTA				
NCDO 2434	CTAGGTG	TTG	GGTGCCAT.A	GGCATTCAG	TGCCGTCGCTA				
NCDO 2398	CTAGGTG	TTG	GGTGCCAT.A	GGCATTCAG	TGCCGTCGCTA				
NCDO 2222	CTAGGTG	TTG	GGTGCCAT.A	GGCATTCAG	TGCCGTCGCTA				
NCDO 2432	CTAGGTG	TTG	GGAGACAT.A	GTTTCTCAG	TGCCGTCGCTA				
Bu 43	CTAGGTG	TTG	GGAACCAC.A	GGTTTTCAG	TGCCGTCGCTA				
NCDO 2221^{T}	CTAGGTG	TTG	GGAGACAT.A	GTCTTTCAG	TGCCGGCGCTA				
E. coli	CTTGGAG	GTT	GTGCCCTTGA	GGCG.TGGC	TTCCGGAGCTA				

b

	450								479
B315	AGAA	CGTGTG	TGAGAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. bovis	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. alactolyticus	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. gallolyticus	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. constellatus	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. intestinalis	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. macedonicus	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. milleri	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. mutans	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. rattus	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. suis	AGAA	CGTGTG	TGACAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. equi	AGAA	CAGTGA	TGGGAGTGGA	AAG	TTCACAC	AGTGACGGTA			
S. acidominimus	AGAA	CGTTAG	CGGGAGTGGA	AAA	TTCACAC	AGTGACGGTA			
E. coli	GGAA	.GGGAG	TAAAGTTAAT	ACC	TTTGCTC	ATTGACGTTA			
B316	AGAA	AG.GCT	CGCAAGA.GA	GA.	.....	TGACGGTA			

3.2.3 Primer Specificity

The primers (B316 830 and B315 454) were used in conjunction with the universal 16S rRNA forward primer fD1* in PCR amplification reactions, and tested for specificity against 85 bacterial strains, mostly of rumen origin (Table 2.1). The B316 830/fD1* primer pair amplified only *C. proteoclasticum* DNA producing a 830 bp PCR product (Fig. 3.4). The B315 454/fD1* primer pair amplified DNA from all *S. bovis* strains tested except lab strain RF–1 (Fig. 3.5). The designation of RF-1 as an *S. bovis* strain is currently under review. Both of these primer pairs produced specific PCR products at 62°C annealing temperature, but at 60°C two unrelated DNA's (*Enterococcus faecalis* strain NCTC 775 and *S. bovis* strain, RF2-B) amplified with the B316 830/fD1* primer pair. This non-specific amplification was eliminated when the temperature was raised to 62°C. To ensure that DNA from each strain was amplifiable, a sample of DNA from each of the rumen bacteria was amplified with the universal primer pair fD1* and rD1* at an annealing temperature of 55°C (Fig 3.6). All strains produced a PCR fragment of approximately 1500 bp. The amplification of *P. ruminicola* 118b and *P. ruminicola* $B_1$4 was repeated to ensure that these bacteria amplified, as the first attempt at amplification produced no amplification of *P. ruminicola* 118b and poor amplification of *P. ruminicola* $B_1$4 (Fig. 3.6).

3.2.4 Internal control construction

The internal controls for both *S. bovis* and *C. proteoclasticum* were constructed by deleting a restriction fragment from the PCR product produced with their respective specific primers. Analysis of the sequence of the *C. proteoclasticum* 830 bp fragment indicated that digestion with *Alu*I restriction endonuclease would produce three fragments of 390, 350 and 90 bp and this was confirmed by sizing *Alu*I restriction fragments in an agarose gel (Fig 3.7). PCR amplification of the religated fragments produced a 480 bp product consistent with the removal of the 350 bp internal fragment. The 480 bp fragment was extracted from the agarose gel and used as an internal control in competitive PCR reactions for *C. proteoclasticum* quantitation. The internal control was amplified with either fD1* or B316 830 primers alone. No amplification occurred

Figure 3.4. B316 830 primer specificity.

DNA extracted from 85 bacteria was amplified with the B316 830 and fD1* primers under standard reaction conditions, 62°C annealing temperature. Each amplification reaction contained 30 ng of template DNA.

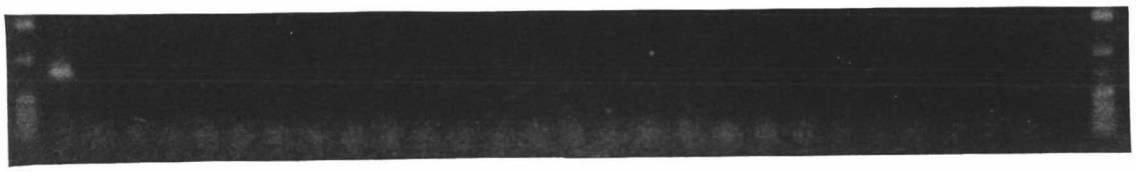
a) lane 1, 1kb molecular weight markers; 2, *C. proteoclasticum*; 3, -DNA; 4, *E. coli*; 5, C21b; 6, C122a; 7, *C. sticklandii*; 8, *C. aminophilum*; 9, *C. clostridioforme* 29084; 10, *C. clostridioforme* 25537; 11, *P. anaerobius*; 12, *P. productus*; 13, *B. fibrisolvens* H17c; 14, *B. fibrisolvens* CF3; 15, *B. fibrisolvens* OR509; 16, *B. fibrisolvens* WV1; 17, C211; 18, C130a; 19, C219; 20, *S. ruminantium* HD4; 21, *S. ruminantium* 195; 22, *S. ruminantium lactilytica*; 23, 10A; 24, *S. amlyolytica*; 25, *S. dextrinosolvens* ATCC 27209; 26 *S. dextrinosolvens* ATCC 19716; 27 *L. multiparus*; 28 ,1 kb molecular weight markers.

b) lane 1, 1 kb molecular weight markers; 2, *C. proteoclasticum*; 3, -DNA; 4, *E. coli*; 5, *S. bovis* JB1; 6, *S. bovis* NCFB 2476; 7, B315; 8, 7-2; 9, 7-25; 10, 7-26; 11, 5-21; 12, 3-31; 13, 3-36; 14, 3-39; 15, A120; 16, B385; 17, B348; 18, B342; 19, B314; 20, B382; 21, B11; 22, B350, 23, B396; 24, C17, 25, A191b; 26, B360; 27, B395; 28, B327; 29, B372; 30, 1 kb molecular weight markers.

c) lane 1, 1 kb molecular weight markers; 2, *C. proteoclasticum*; 3, -DNA; 4, *E. coli*; 5, *P. ruminicola* 118b; 6, *P. rumincola* $B_1$4; 7, *P. ruminicola ruminicola* 23; 8, C21a; 9, *E. cellulosolvens*; 10, *E. limosum*; 11, *E. ruminantium*; 12, C120a; 13, C124b; 14, C12b, 15, C13b, 16, C260; 17, C125b; 18, C14b#2; 19, C118b; 20, C119b; 21, C11; 22, *R. albus* 7; 23, *R. albus* 8; 24, *R, flavefaciens*; 25, *R. flavefaciens* FD1; 26, *M. elsdenii*; 27, *F. succinogenes*; 28, 1 kb molecular weight markers.

d) lane 1, 1 kb molecular weight markers; 2, *C. proteoclasticum* DNA; 3, , -DNA; 4; *E. coli*; 5, B337; 6, B398; 7, A14; 8, C123b; 9, C271; 10, A166; 11, C14b#1; 12, B346; 13, B32a; 14, A12; 15, UDY-KF; 16, RF-1; 17, RF-2; 18, *E. faecalis*; 19, 68a; 20, 1 kb molecular weight markers.

a

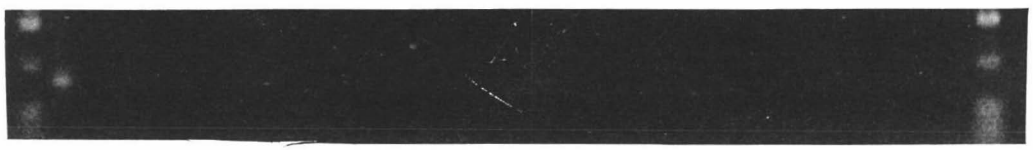


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

b

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

c



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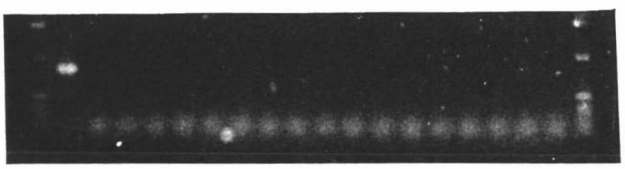


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 3.5. B315 454 primer specificity.

DNA extracted from 85 bacteria was amplified with the B315 445 and fD1* primers under standard conditions at 62°C annealing temperature. Each amplification reaction contained 30 ng of template DNA.

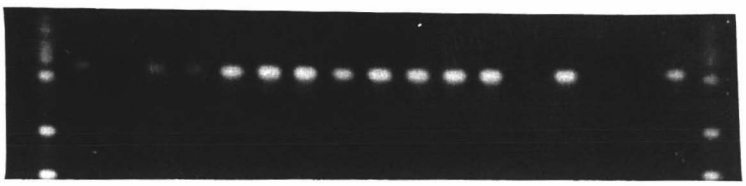
a) lane 1, 1 kb molecular weight markers; 2, B315; 3, -DNA; 4; *E. coli*; 5, B337; 6, B398; 7, A14; 8, C123b; 9, C271; 10, A166; 11, C14b#1; 12, B346; 13, B32a; 14, RF-1; 15, RF-2; 16, *E. faecalis*; 17,;68a 18, UDY-KF; 19, 1 kb molecular weight markers.

b) lane 1, 1 kb molecular weight markers; 2, B315; 3, -DNA; 4, *E. coli*; 5, *S. bovis* JB1; 6, *S. bovis* NCFB 2476; 7,7-2; 8, 7-25; 9, 7-26; 10, 5-21; 11, 3-31; 12, 3-36; 13, 3-39; 14, A12; 15, A120; 16, B385; 17, B348; 18, B342; 19, B314; 20, B382; 21, B11; 22, B350, 23, B396; 24, C17, 25, A191b; 26, B360; 27, B395; 28, B327; 29, B 372; 30, 1 kb molecular weight markers.

c) lane 1, 1 kb molecular weight markers; 2, B315; 3, -DNA; 4, *E. coli*; 5, C21b; 6, C122a; 7, *C. sticklandii*; 8, *C. aminophilum*; 9, *C. clostridioforme* 29084; 10, *C. clostridioforme* 25537; 11, *P. anaerobius*; 12, *P. productus*; 13, *B. fibrisolvens* H17c; 14, *B. fibrisolvens* CF3; 15, *B. fibrisolvens* OR509; 16, *B. fibrisolvens* WV1; 17, C211; 18, C130a; 19, C219; 20, *S. ruminantium* HD4; 21, *S. ruminantium* 195; 22, *S. ruminantium lactilytica*; 23, 10A; 24, *S. amlyolytica*; 25, *S. dextrinosolvens* ATCC 27209; 26 *S. dextrinosolvens* ATCC 19716; 27 *L. multiparus*; 28 ,1 kb molecular weight markers.

d) lane 1, 1 kb molecular weight markers; 2, B315; 3, -DNA; 4, *E. coli*; 5, *P. ruminicola* 118b; 6, *P. rumincola* B_14; 7, *P. ruminicola ruminicola* 23; 8, C21a; 9, *E. cellulosolvens*; 10, *E. limosum*; 11, *E. ruminantium*; 12, C120a; 13, C124b; 14, C12b, 15, C13b, 16, C260; 17, C125b; 18, C14b#2; 19, C118b; 20, C119b; 21, C11; 22, *R. albus* 7; 23, *R. albus* 8; 24, *R, flavefaciens*; 25, *R. flavefaciens* FD1; 26, *M. elsdenii*; 27, *F. succinogenes*; 28 1 kb molecular weight markers.

a



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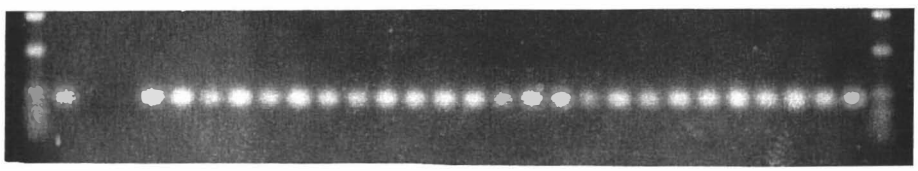


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

c

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

d

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

Figure 3.6. Verification of template DNA.

DNA extracted from 85 bacteria was amplified with the fD1* and rD1* primers under standard conditions at 55°C annealing temperature. Each amplification reaction contained 30 ng of template DNA.

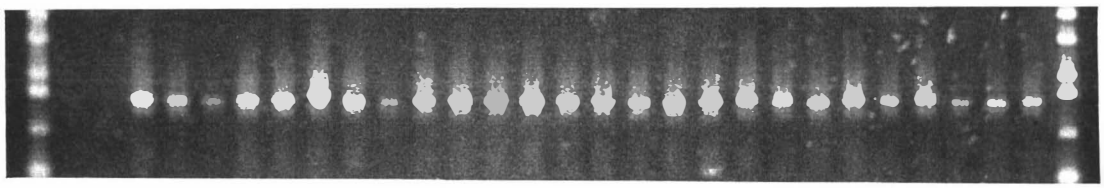
a) lane 1, 1 kb molecular weight markers; 2, *E. coli*; 3, -DNA; 4, *C. proteoclasticum*; 5, C21b; 6, C122a; 7, *C. sticklandii*; 8, *C. aminophilum*; 9, *C. clostridioforme* 29084; 10, *C. clostridioforme* 25537; 11, *P. anaerobius*; 12, *P. productus*; 13, *B. fibrisolvens* H17c; 14, *B. fibrisolvens* CF3; 15, *B. fibrisolvens* OR509; 16, *B. fibrisolvens* WV1; 17, C211; 18, C130a; 19, C219; 20, *S. ruminantium* HD4; 21, *S. ruminantium* 192; 22, *S. ruminantium lactilytica*; 23, 10A; 24, *S. amlyolytica*; 25, *S. dextrinosolvens* ATCC 27209; 26 *S. dextrinosolvens* ATCC 19716; 27 *L. multiparus*; 28 , *P. ruminicola* 118b; 29, *P. rumincola* $B_1$4; 30, 1 kb molecular weight markers.

b) lane 1, 1 kb molecular weight markers; 2, *E. coli*; 3, -DNA; 4, *E. coli*; 5, *P. ruminicola* 118b; 6, *P. rumincola* $B_1$4; 7, *P. ruminicola ruminicola* 23; 8, C21a; 9, *E. cellulosolvens*; 10, *E. limosum*; 11, *E. ruminantium*; 12, C120a; 13, C124b; 14, C12b, 15, C13b, 16, C260; 17, C125b; 18, C14b#2; 19, C118b; 20, C119b; 21, C11; 22, *R. albus* 7; 23, *R. albus* 8; 24, *R, flavefaciens*; 25, *R. flavefaciens* FD1; 26, *M. elsdenii*; 27, *F. succinogenes*; 28 *E. faecalis*; 29, 68a; 30, 1 kb molecular weight markers.

c) lane 1, 1 kb molecular weight markers; 2, *E. coli*; 3, -DNA; 4, B315; 5, *S. bovis* JB1; 6, *S. bovis* NCFB 2476; 7,7-2; 8, 7-25; 9, 7-26; 10, 5-21; 11, 3-31; 12, 3-36; 13, 3-39; 14, A12; 15, A120; 16, B385; 17, B348; 18, B342; 19, B314; 20, B382; 21, B11; 22, B350, 23, B396; 24, C17, 25, A191b; 26, B360; 27, B395; 28, B327; 29, B 372; 30, 1 kb molecular weight markers.

d) lane 1, 1 kb molecular weight markers; 2, *E .coli*; 3, -DNA; 4, B337; 5, B398; 6, A14; 7, C123b; 8, C271; 9, A166; 10, C14b#1; 11, B346; 12, B32a; 13, UDY-KF; 14, RF-1; 15, RF-2; 16, 1 kb molecular weight markers.

a



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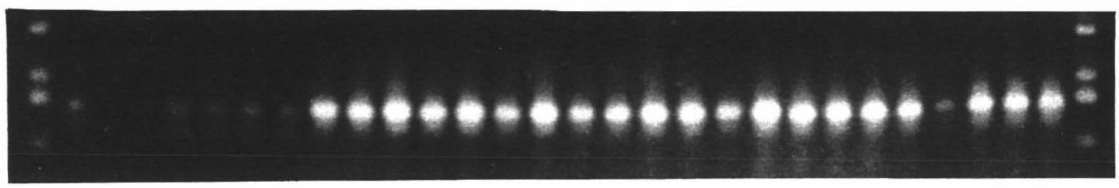

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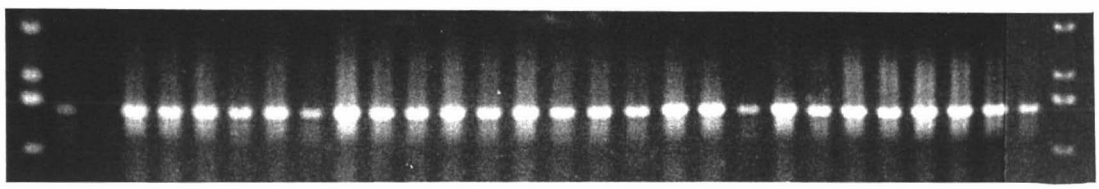

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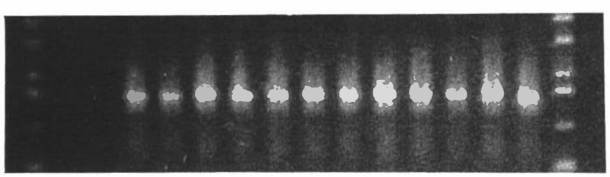
d



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

unless both forward and reverse primers were present, confirming that both priming sites were present and that the fragments had ligated together correctly.

Digestion of the *S. bovis* 454 bp fragment with *Alu*I resulted in five fragments of 44, 73, 86, 95 and 175 bp which were predicted from analysis of the sequence (Fig. 3.8). PCR amplification of the ligated restriction fragments resulted in three PCR products of 203, 161 and 117 bp (Fig. 3.8). The 203 bp fragment results from the ligation of the 73 bp fragment containing the fD1* primer binding site, the 86 bp internal fragment and the 44 bp fragment containing the B315 454 primer binding site. The 117 bp fragment is comprised of the 44 and 73 bp fragments containing the respective primer binding sites. The 161 bp fragment is most likely a ligation of the 73 bp fragment ligated with two 44 bp fragments each containing the B315 454 primer binding site. Upon amplification of the 161 bp ligation product, two products could possibly result, a 117 bp product and a 161 bp product. This explains the observation that the 117 bp band is twice the intensity of the 161 bp band as seen in the agarose gel (Fig. 3.8). As the 117 bp band is the simplest ligation product and is sufficiently different in size to be easily distinguished from the 454 bp fragment it was used for competitive PCR quantitation of *S. bovis*. The *S. bovis* internal control was also amplified with either the fD1* or B315 454 primer alone, and no amplification resulted indicating the presence of both priming sites.

3.2.5 Internal control amplification efficiency

Accurate quantitation using competitive PCR requires that the internal control DNA and the target sequence in the genomic DNA amplify with equal efficiency. This can be determined by plotting the log ratio (target intensity/internal control intensity) against the log concentration of the internal control (Zachar *et al.*,1993). Dilutions of *C. proteoclasticum* internal control DNA were co-amplified with DNA from the equivalent of 1 x 10^3 *C. proteoclasticum* cells (Fig. 3.9), and the intensities of target and internal control used to plot their amplification efficiencies. The results show a line with a slope of 0.94, and a regression of 0.99. This indicates that the amplification efficiencies of the internal control DNA and the target *C proteoclasticum* sequence are essentially equal.

Figure 3.7. Construction of the *C. proteoclasticum* internal control.

The 830 bp PCR product was digested with *Alu*I to give three fragments of 90, 350 and 390 bp. The three fragments were ligated and PCR amplified. The 480 bp product was purified from agarose and used directly as the internal control in cPCR.

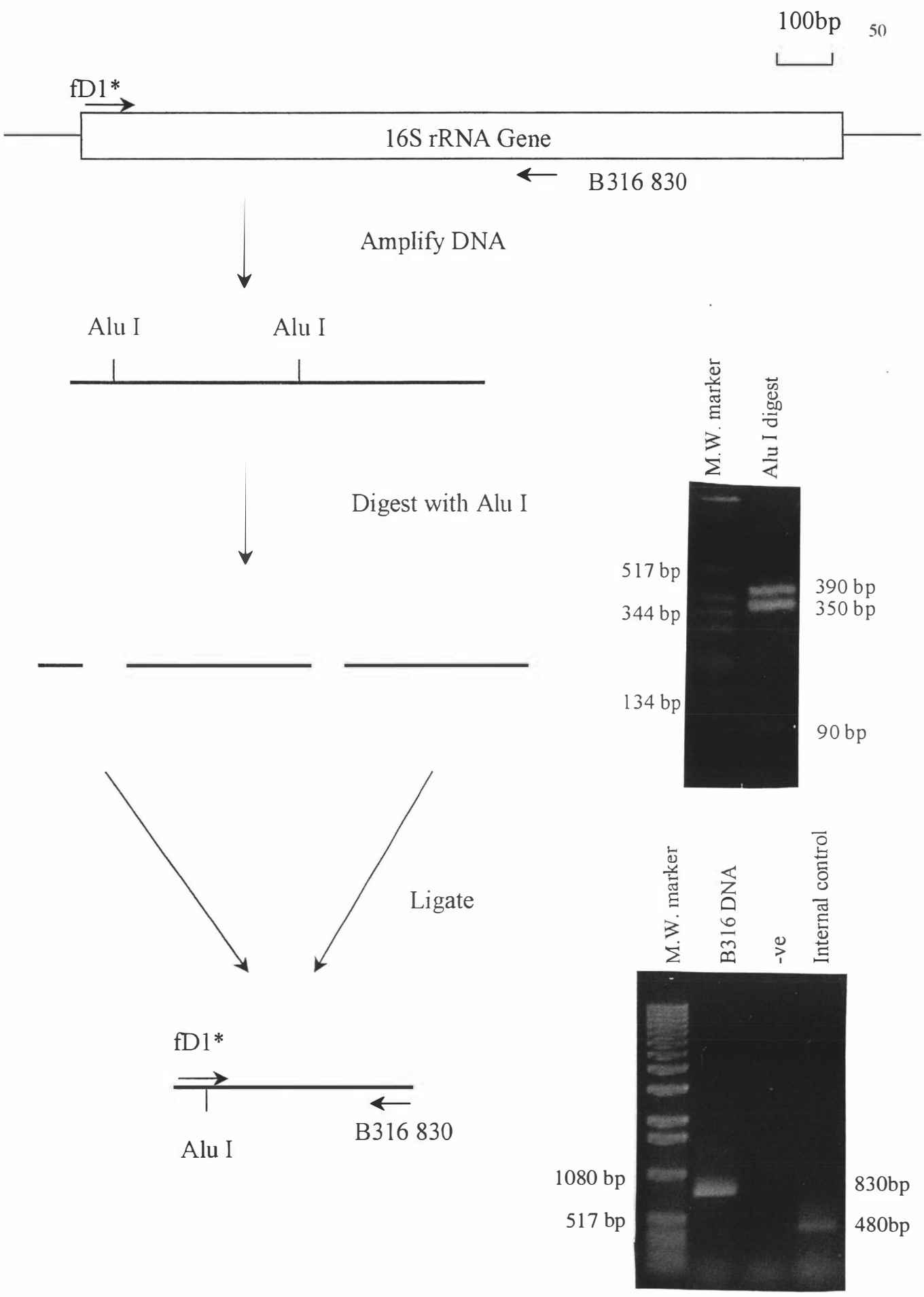
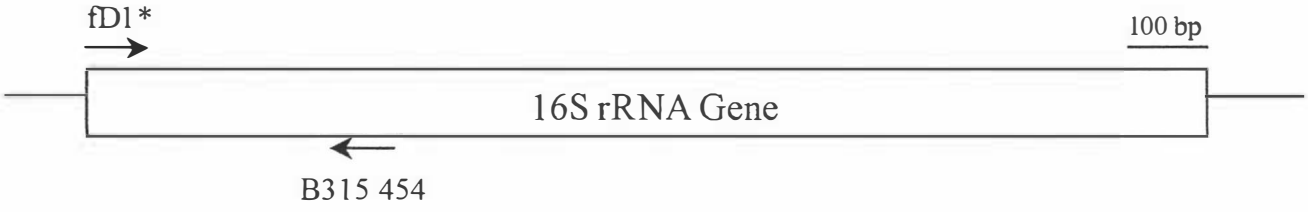
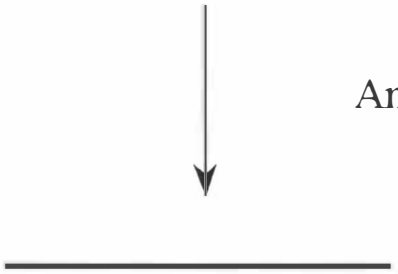
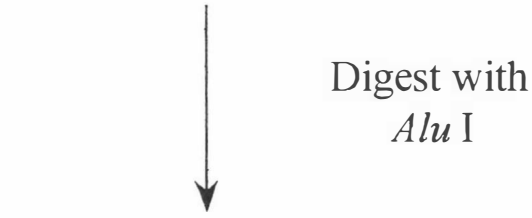

100bp
50
fD1*
16S rRNA Gene
B316 830
Amplify DNA
Alu I
Alu I
Digest with Alu I
M.W. marker
Alu I digest
517 bp
344 bp
134 bp
390 bp
350 bp
90 bp
Ligate
fD1*
B316 830
Alu I
M.W. marker
B316 DNA
-ve
Internal control
1080 bp
517 bp
830bp
480bp

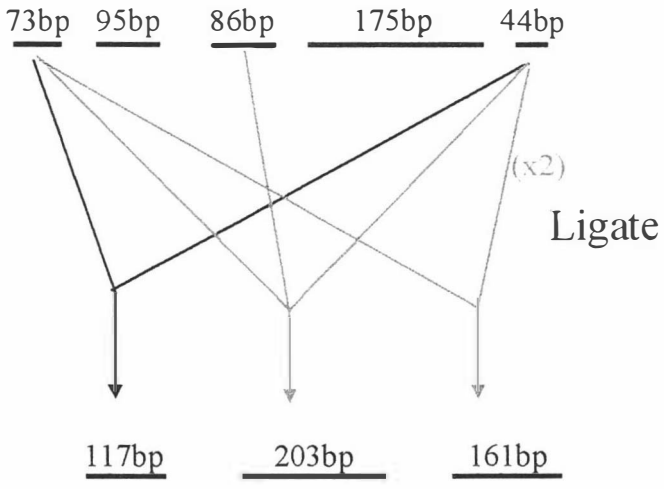
Figure 3.8. Construction of the *Streptococcus* internal control.

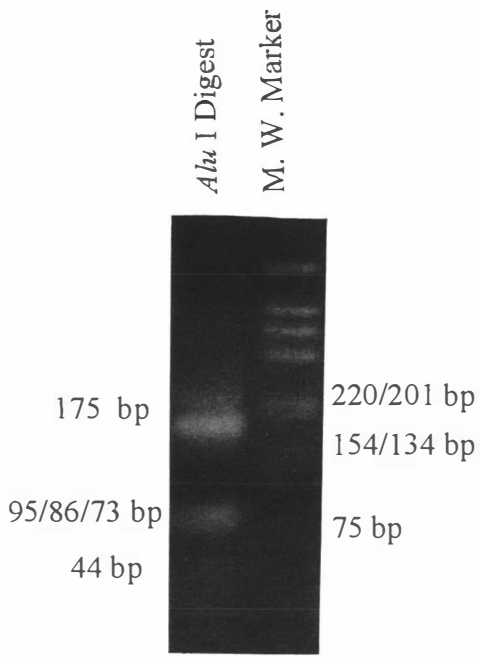
The 454 bp PCR product was digested with *Alu*I to give five fragments of 44, 73, 86, 95 and 175 bp. The five fragments were ligated and PCR amplified, producing three amplification products, of 117, 161, and 201 bp in size. The 117 bp fragment was isolated from the gel and used as the *Streptococcus* internal control (lane 4).


fD1*
100 bp
16S rRNA Gene
B315 454


Amplify


Digest with
Alu I


73bp
95bp
86bp
175bp
44bp
(x2)
Ligate
117bp
203bp
161bp


Alu I Digest
M. W. Marker
175 bp
95/86/73 bp
44 bp
220/201 bp
154/134 bp
75 bp

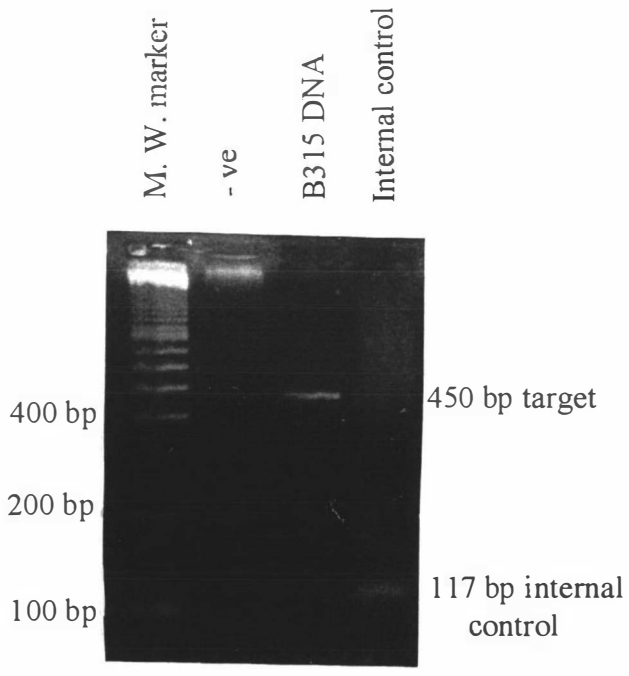
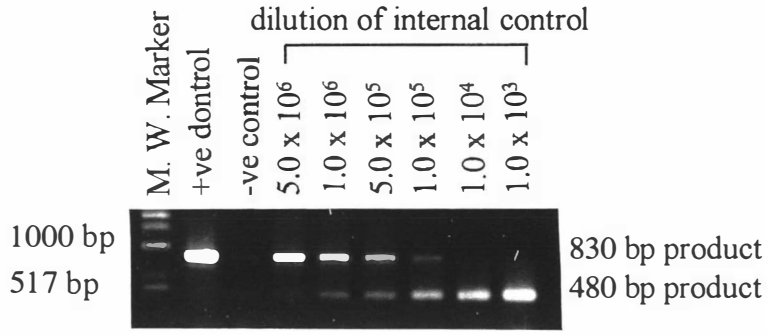

M. W. marker
- ve
B315 DNA
Internal control
400 bp
200 bp
100 bp
450 bp target
117 bp internal control

Figure 3.9. *C. proteoclasticum* internal control amplification efficiency.

a) Dilutions of the *C. proteoclasticum* internal control were co-amplified with *C. proteoclasticum* DNA (equivalent to 1 x 10^3 *C. proteoclasticum* cells).

b) The intensities of internal control DNA and target DNA were quantitated by scanning densitometry of negative images of Polaroid photographs of ethidium bromide stained gels. The ratio of the intensities of the internal control to target DNA was plotted against the dilution of the internal control on a log scale.

a


dilution of internal control
M. W. Marker
+ve dontrol
-ve control
5.0 x 10⁶
1.0 x 10⁶
5.0 x 10⁵
1.0 x 10⁵
1.0 x 10⁴
1.0 x 10³
1000 bp
517 bp
830 bp product
480 bp product

b

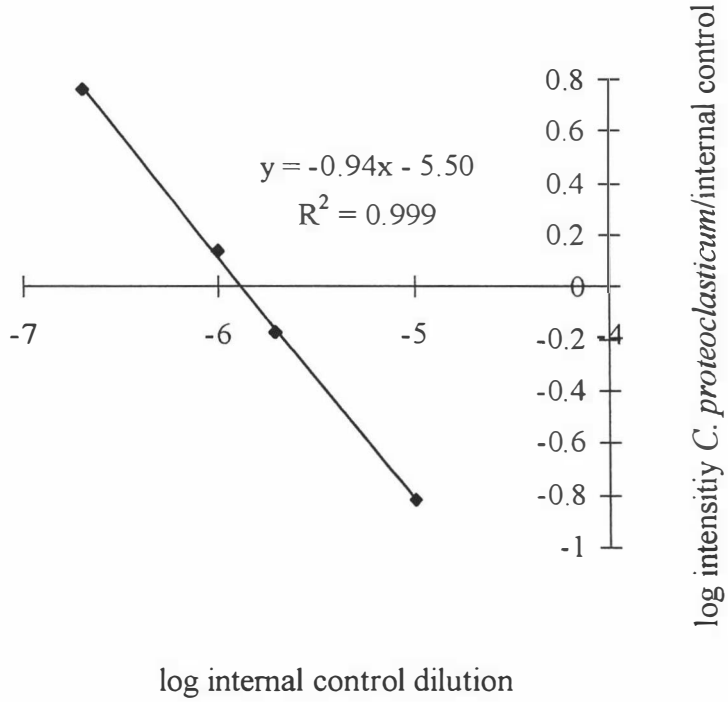
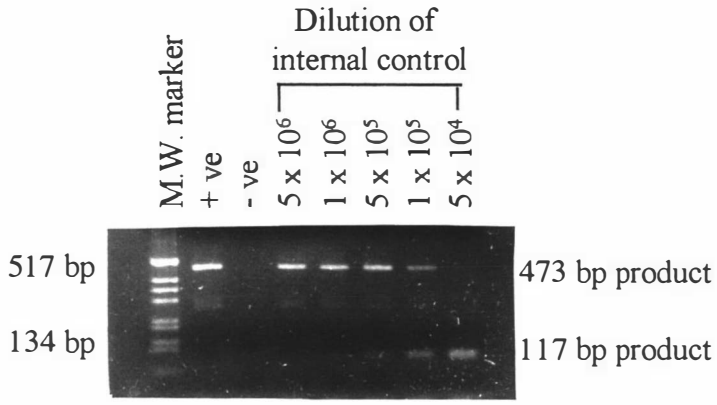

y = -0.94x - 5.50
R² = 0.999
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-7
-6
-5
-4
log intensitiy C. proteoclasticum/internal control
log internal control dilution

Figure 3.10. *S. bovis* internal control amplification efficiency.

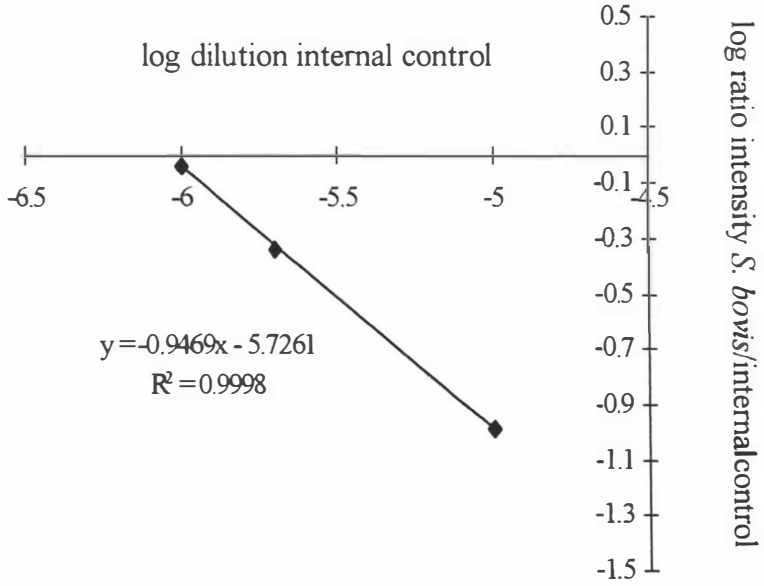
a) Dilutions of the *S. bovis* internal control were co-amplified with DNA equivalent to 1 x 10^3 *S. bovis* cells.

b) The intensities of internal control DNA and target DNA were quantitated by scanning densitometry of negative images of Polaroid photographs of ethidium bromide stained gels. The ratio of the intensities of the internal control to target DNA was plotted against the dilution of the internal control on a log scale.

a


Dilution of
internal control
M.W. marker
+ ve
- ve
5 x 10⁶
1 x 10⁶
5 x 10⁵
1 x 10⁵
5 x 10⁴
517 bp
134 bp
473 bp product
117 bp product

b


log dilution internal control
log ratio intensity S. bovis/internalcontrol
-6.5
-6
-5.5
-5
-4.5
0.5
0.3
0.1
-0.1
-0.3
-0.5
-0.7
-0.9
-1.1
-1.3
-1.5
y = -0.9469x - 5.7261
R² = 0.9998

Similar experiments were carried out with *S. bovis* DNA and its internal control, and the results show a line with a slope of 0.95, and a regression of 0.99 (Fig 3.10), again indication equivalent amplification of target and internal control DNA.

3.3 Discussion

16S rDNA analysis of *B. fibrisolvens*-like strain B316 led to its classification as a novel species, *Clostridium proteoclasticum* (Attwood *et al.,* 1996). Initial alignments of the 16S rRNA gene identified *C. aminophilum* as the most closely related bacterium with 92.2% similarity (Attwood *et al*, 1996). However, since this description, publication of 16S rDNA sequences of *B. fibrisolvens* strains NCDO 2435, 2434, 2222, 2342 and 2398 has revealed that these organisms are closely related to *C. proteoclasticum* (96.9 to 99.5% homology, Fig. 3.1). However, the 16S rRNA sequence of the *B. fibrisolvens* type strain D1 (NCDO 2221^T) is only distantly related to these organisms (93.1 to 94.1%) therefore the classification of *C. proteoclasticum* as a separate species remains justified.

The three *S. bovis* strains chosen for this investigation (local strains B315, C14b#1 and NCFB strain 2476) have slightly different phenotypic characteristics, and the possibility that their 16S rRNA sequences would reflect this divergence was investigated. Based on their 16S rDNA sequences these organisms were closely related to each other (99.7-99.9%) and also to the *S. bovis* type strain NCDO 597 (99.5%) used in the phylogenetic analysis (Fig 3.2), thus confirming these isolates as strains of *S. bovis.* However the 16S rDNA gene proved unsuitable for determining a meaningful relationship between these closely related strains. A more detailed study at the molecular level such as DNA/DNA hybridisation may be a more suitable method for determining the phylogenetic relationships between these strains (Stackebrandt and Goebel, 1994).

The V3 hyper variable region of *S. bovis* and V5 hyper variable region from *C. proteoclasticum* 16S rRNA genes were used to design PCR primers specific for each of these bacteria. The specific primers were designed complimentary to the sense strand (ie. reverse direction) of the hyper variable region within the 16S rDNA chosen for

either *C. proteoclasticum* or *S. bovis*. The primers were designed to match as closely as possible, the universal forward primer, fD1*, in length, % G + C, and T_m. The specific primers were used in conjunction with the universal primer fD1* which anchored the PCR reactions at the 5' end of the gene. This approach limits the primers that can be designed within the 16S rRNA gene, but means only one primer needs to be designed for each target organism. Using the 16S gene as a target for primer design also enables screening of primers against a the sequences in the ribosomal database (Olsen *et al*, 1993). The fD1* and B316 830 primer pair did not amplify any DNA from any organism tested except *C. proteoclasticum*. A BLAST search of the B316 830 primer revealed that it was 100% homologous to a region in the 16S rDNA sequences of the four closely related *B. fibrisolvens* strains NCDO 2435 2343 2222 and 2398. However B316 830 does not share exactly the same sequence in the corresponding genes of *B. fibrisolvens* strains Bu 43, NCDO 2432 or $D1^T$ (Fig. 3.3), and have 5, 6 and 4 base pair differences respectively. The differences of these sites in the *B. fibrisolvens* type strain (D1, NCDO 2221^T) and strain Bu 43 are consistent with their phylogenetic position in relation to the rest of this *B. fibrisolvens* cluster (Fig. 3.2). However, the differences at the same site in strain NCDO 2432 do not appear to be consistent with the same analysis. In 16S rDNA analyses, the hyper-variable regions, such as the target site for the B316 830 primer, are excluded from the comparisons as they are often too variable to produce meaningful relationships. In this case, the variation observed at the variable site in strain NCDO 2432 may indicate greater phylogenetic diversity than is apparent from the analysis of the conserved 16S rDNA regions. Therefore the *C. proteoclasticum* B316 830 primer seems to circunscribe a subset of the this *B. fibrisolvens* cluster and will useful for determining populations of these strains.

The fD1* and B315 454 primer pair amplified DNA from all strains of *S. bovis* tested, but no other bacterium. By BLAST search the *S. bovis* B315 454 primer had 100% homology with *S. constellatus, S. mutans, S. equinus, S. gallolyticus, S. macedonicus, S. intestinalis, S, alactolyticus, S. rattus, S. suis* and *S. milleri* (Fig 3.3) as well as published *S. bovis* sequences. These bacteria do not form a particular cluster among the *Streptococci* and appear randomly distributed in terms of evolution. However, of these isolates only *S. caprinus* and *S. bovis* has been isolated from the rumen. Therefore the B315 454 primer is probably a *Streptococcus* genus level primer for use in the rumen ecosystem.

Design and construction of effective internal control DNAs are central to successful cPCR. Many difference methods have been employed for internal control construction including the deletion or insertion of sequences between priming sites, looped oligos or the incorporation of novel restriction enzyme sites (Leser, 1995; Lee *et al.,* 1996; Kobayashi *et al.,* 1998). In this study we chose to delete internal *Alu*I restriction fragments from the target sequences of both *C. proteoclasticum* and *S. bovis* to generate internal control DNAs of 480 bp and 117 bp respectively. The construction of the *C. proteoclasticum* internal control was straight forward, with only two possible ligation products that were able to amplify: the original intact fragment, or the deletion fragment. However the four *Alu*I restriction sites in the *S. bovis* PCR fragment resulted in five restriction fragments. After religation three amplification products were observed, of which only two were easily explainable. Having multiple amplification fragments present after ligation also presented the possibility of contamination when excising the internal control DNA from the agarose gel. These problems could be overcome by excising the relevant bands after restriction digestion and ligating them under controlled conditions so fewer ligation products are possible. Alternatively, cloning of the intact PCR fragment prior to digestion would also aid the recombining of fragments in a predictable manner, and would also facilitate the use of blunt ending for incompatible restriction enzymes

In order to quantitate accurately using cPCR, it is important that the internal control DNA amplifies with the same amplification efficiency as the target DNA. The relative amplification efficiencies of the target DNA and the internal control determined for *C. proteoclasticum* and *S. bovis* indicated essentially equivalent amplification. In both cases the amplification efficiencies of the internal controls were essentially the same as the target DNA. The internal control DNA is slightly more efficient than the target DNA, which could be due to smaller size of the internal controls. As pointed out by Zacher *et al.* (1993), the assumption that the concentration of PCR end products is proportional to the amount of starting products is still valid if the efficiency of the reactions are not equal, as long as eff_1/eff_2 is constant and amplification is in the exponential phase.

Quantitation of bacteria from rumen samples has long been fraught with difficulties. We have developed a PCR primer that is specific for *C. proteoclasticum* and closely related *B. fibrisolvens* strains and a PCR primer specific for streptococci in the rumen. These PCR primers will differentiate these strains from the mixed microbial population, and in conjunction with the internal control DNA's facilitate the enumeration of these bacteria in the rumen ecosystem using a cPCR based system.

Chapter 4 - ENUMERATION OF *C. proteoclasticum* AND STREPTOCOCCI FROM RUMEN SAMPLES BY COMPETITIVE PCR

4.1 INTRODUCTION

The rumen ecosystem is a diverse environment. Quantitation of bacteria from this microcosm using traditional culturing techniques is not reliable, and there is an inability to culture all the micro-organisms observed under the microscope (Amann *et al.,* 1995). Molecular techniques avoid some of these problems by analysing nucleic acids directly from the environment directly. Competitive PCR (cPCR) is a technique which combines the specificity of 16S rRNA probes with the sensitivity of PCR, and has been used to quantitate bacterial populations from environmental samples previously (Leser *et al.,* 1995; Lee *et al.,* 1996; Kobayashi *et al.*, 1998). The purpose of this study was to use cPCR to enumerate *C. proteoclasticum* and *Streptococci* directly from rumen samples. In order to quantitate using cPCR, standard curve construction was undertaken to relate the ratio of target/internal control band intensity to cell numbers, the most efficient method of DNA extraction was investigated and the detection limit of cPCR for *C. proteoclasticum* and *S. bovis* was determined.

4.2 RESULTS

4.2.1 Co-amplification detection limit

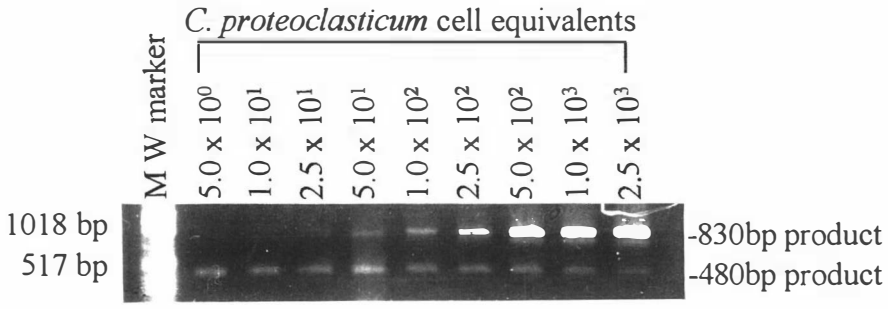
The detection limit of the cPCR technique was examined by co-amplification of a serial dilution of DNA extracted from a known number of cells with increasing dilutions of the internal control. The sensitivity of detection for both *S. bovis* and *C. proteoclasticum* was the DNA from 25 cell equivalents (Fig. 4.1) in each reaction. A 5 x 10^6 dilution of the internal control was required for the detection of 25 *S. bovis* cells, while a 2 x 10^6 dilution of the internal control was required to detect *C. proteoclasticum* (Fig. 4.1).

Figure 4.1. Detection limit of cPCR.

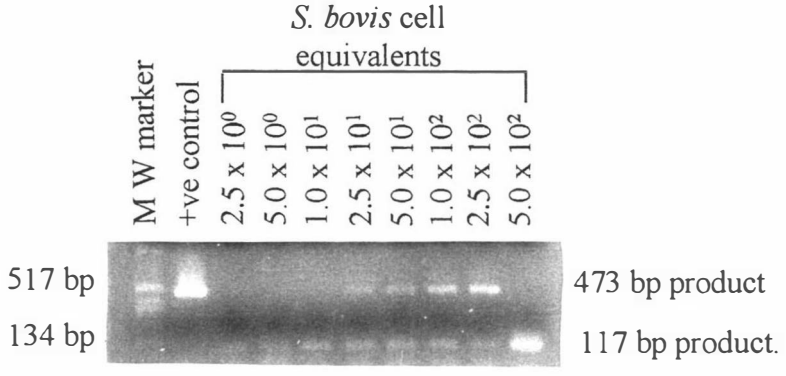
a) DNA extracted from 1 x 10^{10} *C. proteoclasticum* cells ml^{-1} was serially diluted and co-amplified with 2 x 10^{6} dilution of *C. proteoclasticum* internal control.

b) DNA extracted from 1 x 10^{10} *S. bovis* cells ml^{-1} was serially diluted and co-amplified with a 5 x 10^{6} dilution of the *Streptococcus* internal control. These results are expressed as cell equivalents based on the amount of DNA extracted per cell.

a


C. proteoclasticum cell equivalents
M W marker
5.0 x 10^0
1.0 x 10^1
2.5 x 10^1
5.0 x 10^1
1.0 x 10^2
2.5 x 10^2
5.0 x 10^2
1.0 x 10^3
2.5 x 10^3
1018 bp
517 bp
-830bp product
-480bp product

b


S. bovis cell equivalents
M W marker
+ve control
2.5 x 10^0
5.0 x 10^0
1.0 x 10^1
2.5 x 10^1
5.0 x 10^1
1.0 x 10^2
2.5 x 10^2
5.0 x 10^2
517 bp
134 bp
473 bp product
117 bp product.

4.2.2 DNA extraction efficiency

In order to determine the most efficient method of DNA extraction from bacterial samples the DNA from 1 x 10^{10} *C. proteoclasticum* cells was extracted by three different methods as listed in section 2.8: chemical extraction with DNAzol reagent followed by bead beating; enzymatic lysis with lysozyme and SDS, and physical disruption by bead beating. Quantitative measurements of the extracted DNA using absorbance readings at 260nm demonstrated that physical disruption was the most efficient method of DNA extraction with a total of 67µg extracted from 1 x 10^{10} *C. proteoclasticum* cells. Chemical extraction and enzymatic lysis recovered 1.0 and 43 µg of DNA respectively.

4.2.3 Standard curve construction

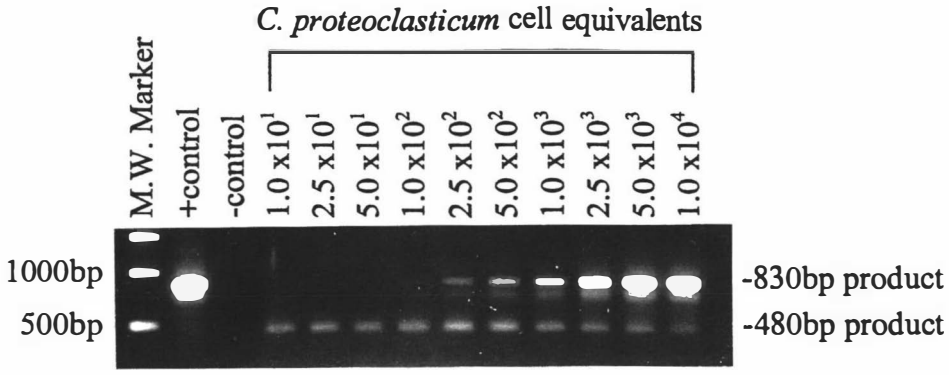
As cPCR is most accurate when the intensities of the internal control and the target sequence are equal, it was necessary to determine the optimal internal control concentration which would co-amplify with DNA extracted from rumen contents. Dilutions of internal control were co-amplified with DNA extracted from rumen contents, and the optimal dilution of internal control was found to be 1 x 10^6 for *C. proteoclasticum* internal control and 1 x 10^5 for the majority of *S. bovis* samples. However, some rumen samples (cows 727, 2a, 2b, 2c; 9754 2a, 2b, 2c; and 1758, all samples) required a 1 x 10^6 dilution of the internal control for amplification. The optimal dilutions of the internal control (1 x 10^6, for *C. proteoclasticum* and both 1 x 10^5 and 1 x 10^6 for *S. bovis*) were co amplified with serial dilutions of DNA extracted from a known number of either *C. proteoclasticum* cells (Fig. 4.2) or *S. bovis* (Fig. 4.3 and 4.4). The results showed a linear response between the ratio of target/internal control band intensities to DNA equivalent to 1 x 10^4 to 5 x 10^1 *C. proteoclasticum* cells. This meant that the 1 x 10^6 dilution of *C. proteoclasticum* internal control could be used for quantitation of samples in this range. The 1 x 10^5 and 1 x 10^6 dilutions of *S. bovis* internal control gave a linear response between ratio of target/internal control intensity and cell number with 2.5 x 10^2 – 5 x ^{4} and 5 x 10^1 – 1 x 10^4 *S. bovis* cells respectively.

Figure 4.2. *C. proteoclasticum* standard curve construction.

a) DNA extracted from 1 x 10^{10} *C. proteoclasticum* cells ml^{-1} was serially diluted and co-amplified with a $1x10^{6}$ dilution of the internal control. The results are expressed as *C. proteoclasticum* cell equivalents based on the amount of DNA extracted per cell.

b) The intensities of internal control DNA and target DNA were quantitated by scanning densitometry of negative images of Polaroid photographs of ethidium bromide stained gels. The ratio of the intensities of the internal control to target DNA was plotted against the dilution of the internal control on a log scale.

a


C. proteoclasticum cell equivalents
M.W. Marker
+control
-control
1.0 x10¹
2.5 x10¹
5.0 x10¹
1.0 x10²
2.5 x10²
5.0 x10²
1.0 x10³
2.5 x10³
5.0 x10³
1.0 x10⁴
1000bp
500bp
-830bp product
-480bp product

b

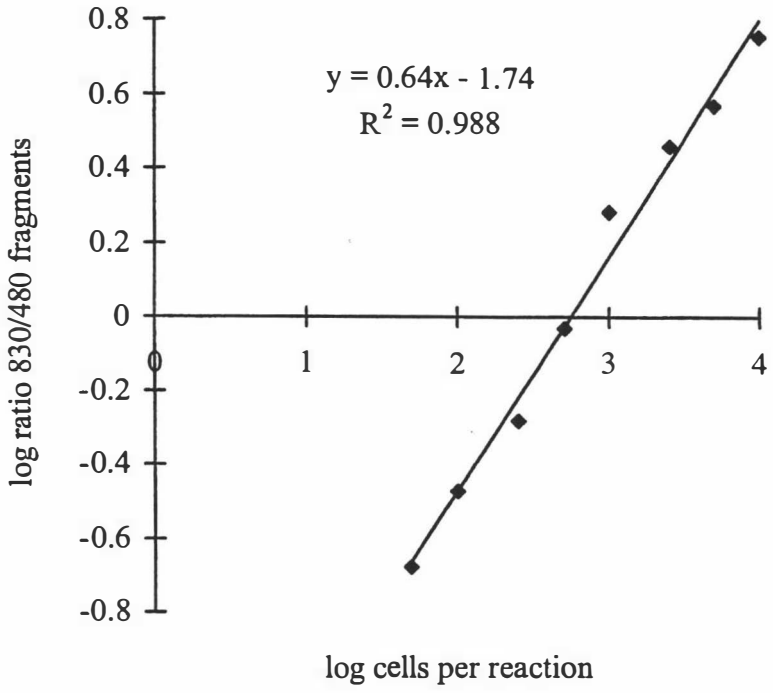
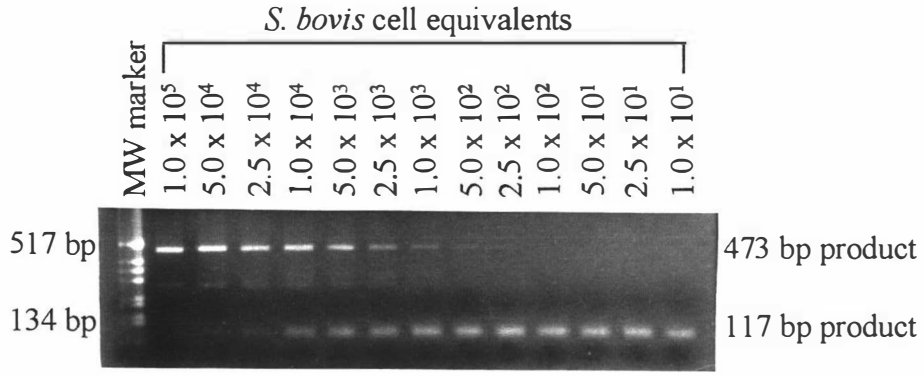

y = 0.64x - 1.74
R² = 0.988
log ratio 830/480 fragments
log cells per reaction
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
0
1
2
3
4

Figure 4.3. *S. bovis* standard curve construction.

a) DNA extracted from 1 x 10^{10} *S. bovis* cells ml^{-1} was serially diluted and co-amplified with a $1x10^{5}$ dilution of the internal control. The results are expressed as *S. bovis* cell equivalents based on the amount of DNA extracted per cell.

b) The intensities of internal control DNA and target DNA were quantitated by scanning densitometry of negative images of Polaroid photographs of ethidium bromide stained gels. The ratio of the intensities of the internal control to target DNA was plotted against the dilution of the internal control on a log scale.

a


S. bovis cell equivalents
MW marker
1.0 x 10^5
5.0 x 10^4
2.5 x 10^4
1.0 x 10^4
5.0 x 10^3
2.5 x 10^3
1.0 x 10^3
5.0 x 10^2
2.5 x 10^2
1.0 x 10^2
5.0 x 10^1
2.5 x 10^1
1.0 x 10^1
517 bp
134 bp
473 bp product
117 bp product

b

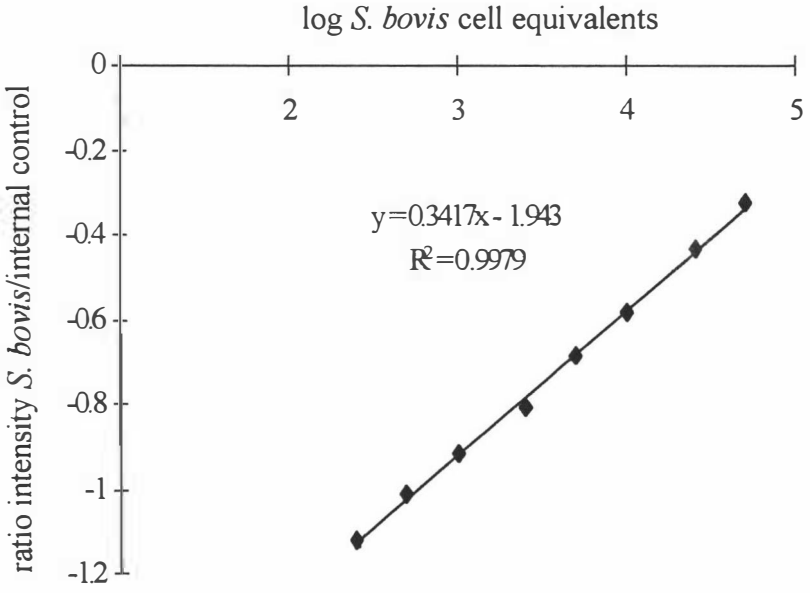
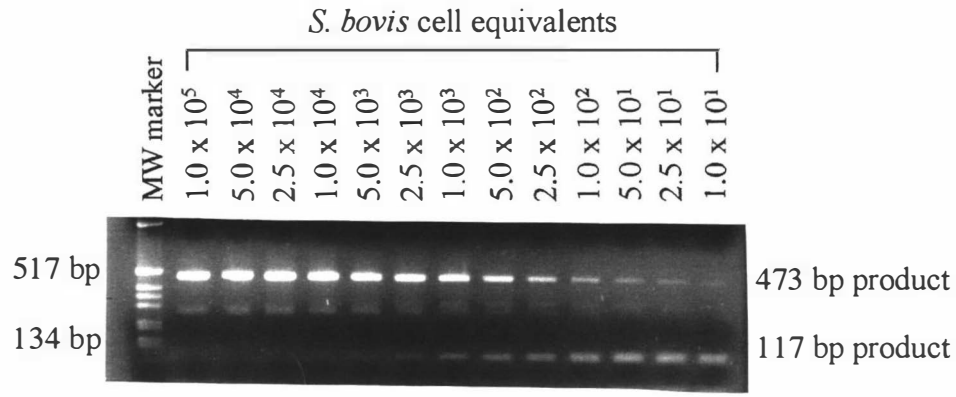

log S. bovis cell equivalents
ratio intensity S. bovis/internal control
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
2
3
4
5
y=0.3417x - 1.943
R²=0.9979

Figure 4.4. *Streptococcus* standard curve construction.

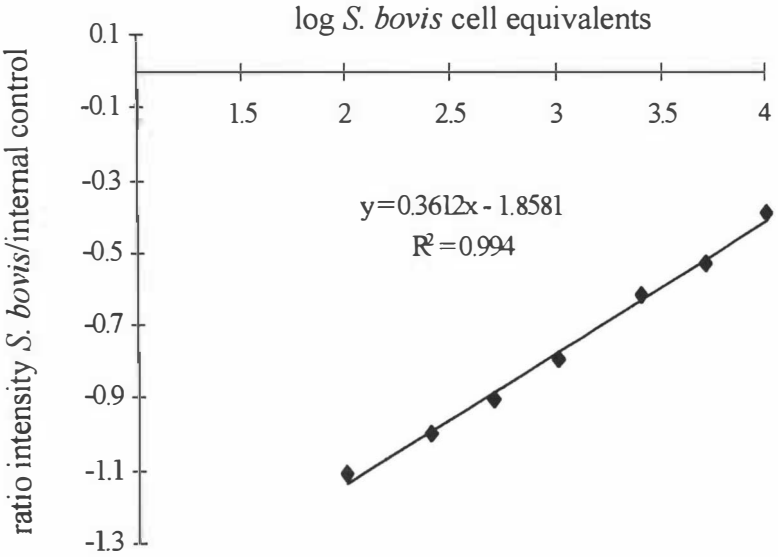
a) In order to co-amplify *Streptococcus* from all rumen samples, a second standard curve was required. DNA extracted from 1 x 10^{10} *S. bovis* cells ml^{-1} was serially diluted and co-amplified with a $1x10^{6}$ dilution of the internal control. The results are expressed as *S. bovis* cell equivalents based on the amount of DNA extracted per cell.

b) The intensities of internal control DNA and target DNA were quantitated by scanning densitometry of negative images of Polaroid photographs of ethidium bromide stained gels. The ratio of the intensities of the internal control to target DNA was plotted against the dilution of the internal control on a log scale.

a


S. bovis cell equivalents
MW marker
1.0 x 10^5
5.0 x 10^4
2.5 x 10^4
1.0 x 10^4
5.0 x 10^3
2.5 x 10^3
1.0 x 10^3
5.0 x 10^2
2.5 x 10^2
1.0 x 10^2
5.0 x 10^1
2.5 x 10^1
1.0 x 10^1
517 bp
134 bp
473 bp product
117 bp product

b


log S. bovis cell equivalents
ratio intensity S. bovis/internal control
0.1
-0.1
-0.3
-0.5
-0.7
-0.9
-1.1
-1.3
1.5
2
2.5
3
3.5
4
y=0.3612x - 1.8581
R² = 0.994

4.2.4 Detection of *C. proteoclasticum* added to rumen fluid

To test whether *C. proteoclasticum* could be detected by cPCR within a background of non-specific DNA from rumen fluid, samples of rumen contents were spiked with known numbers of *C. proteoclasticum* cells. DNA extracted from these samples was co amplified with the 1 x 10^6 dilution of the *C. proteoclasticum* internal control, and the numbers of *C. proteoclasticum* were determined (Fig. 4.4). The results show a linear response between the number of cells added and the number of cells detected. When compared to the theoretical ideal of y = x, the assay slightly overestimated the numbers of *C. proteoclasticum* present in the rumen samples, and this is more pronounced in the rumen samples containing the higher populations of *C. proteoclasticum.* A background population of 6.25 x 10^6 cells was detected in the unspiked samples.

4.2.5 Detection of *C. proteoclasticum* and *S. bovis in vivo*

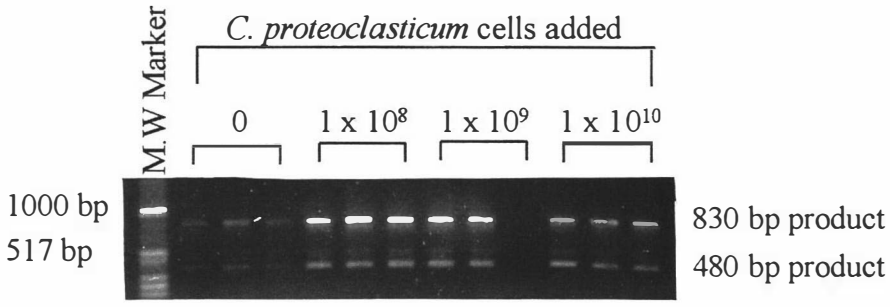
To examine the application of cPCR to determining bacterial numbers directly from animals and to assess the inpact of nitrogen and carbohydrate additions on the populations of *C. proteoclasticum* and *S. bovis in vivo*, rumen samples were collected from eight lactating Fresian dairy cows fed four different diets in rotation: high nitrogen; high nitrogen with carbohydrate; low nitrogen and low nitrogen with carbohydrate. DNA extracted from the rumen samples was also amplified with the universal primers to ensure that DNA was present and that it was PCR amplifiable. Rumen samples were spiked with the appropriate dilution of the internal control, followed by amplification and quantitation. The populations of *C. proteoclasticum* detected in animals on the different diets ranged from 2.06 x 10^6 to 3.12 x 10^7 cells per ml (Fig. 4.5) while *S. bovis* ranged from 1.17 x 10^7 to 1.33 x 10^8 bacterial cells per ml (Fig. 4.6). There was no significant difference between any of the diets for either bacterial population.

The enumeration of *C. proteoclasticum* from rumen samples by cPCR has recently been published (Reilly and Attwood, 1998). A copy of this paper is included in appendix D.

Figure 4.5. *In vivo* detection of *C. proteoclasticum.*

Rumen samples were spiked with known numbers of *C. proteoclasticum* cells. DNA was extracted from these mixtures and assayed for the presence of *C. proteoclasticum.* Closed squares denote numbers of *C. proteoclasticum* detected in each sample. Open circles denote y = x. The y intercept of the line denotes no *C. proteoclasticum* cells added. This represents the background *C. proteoclasticum* population of 6.46×10^6 ml^{-1}.

a


C. proteoclasticum cells added
M.W Marker
0
1 x 10^8
1 x 10^9
1 x 10^10
1000 bp
517 bp
830 bp product
480 bp product

b

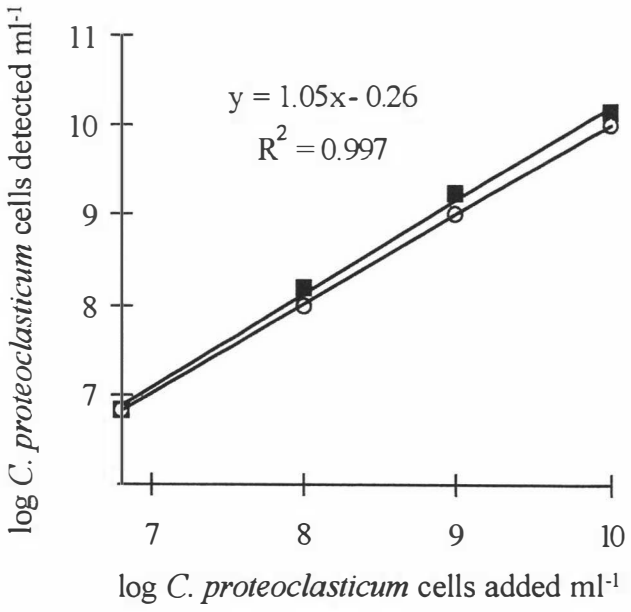

y = 1.05x- 0.26
R^2 = 0.997
log C. proteoclasticum cells detected ml^-1
log C. proteoclasticum cells added ml^-1
11
10
9
8
7
7
8
9
10

Figure 4.6. Populations of *C. proteoclasticum* in dairy cows under four different feeding regimes.

Numbers 1 through 8 represent cows numbered 709, 710, 727, 788, 1758, 8702, 9754 and 9775 respectively. Results are the means of triplicate determinations and error bars represent standard error of the mean. The diets were fed in rotation and rumen samples were taken during each feeding regime.

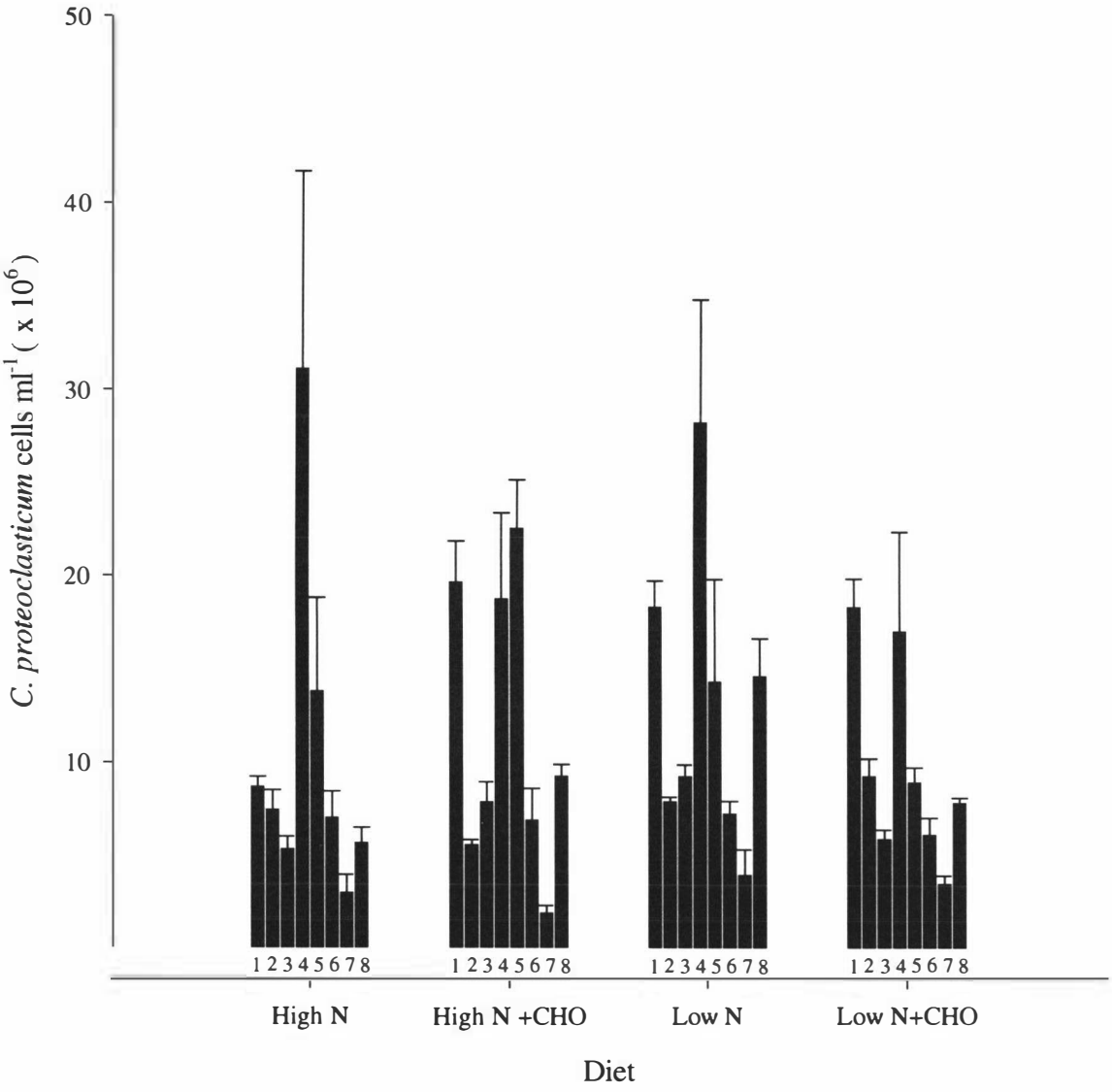
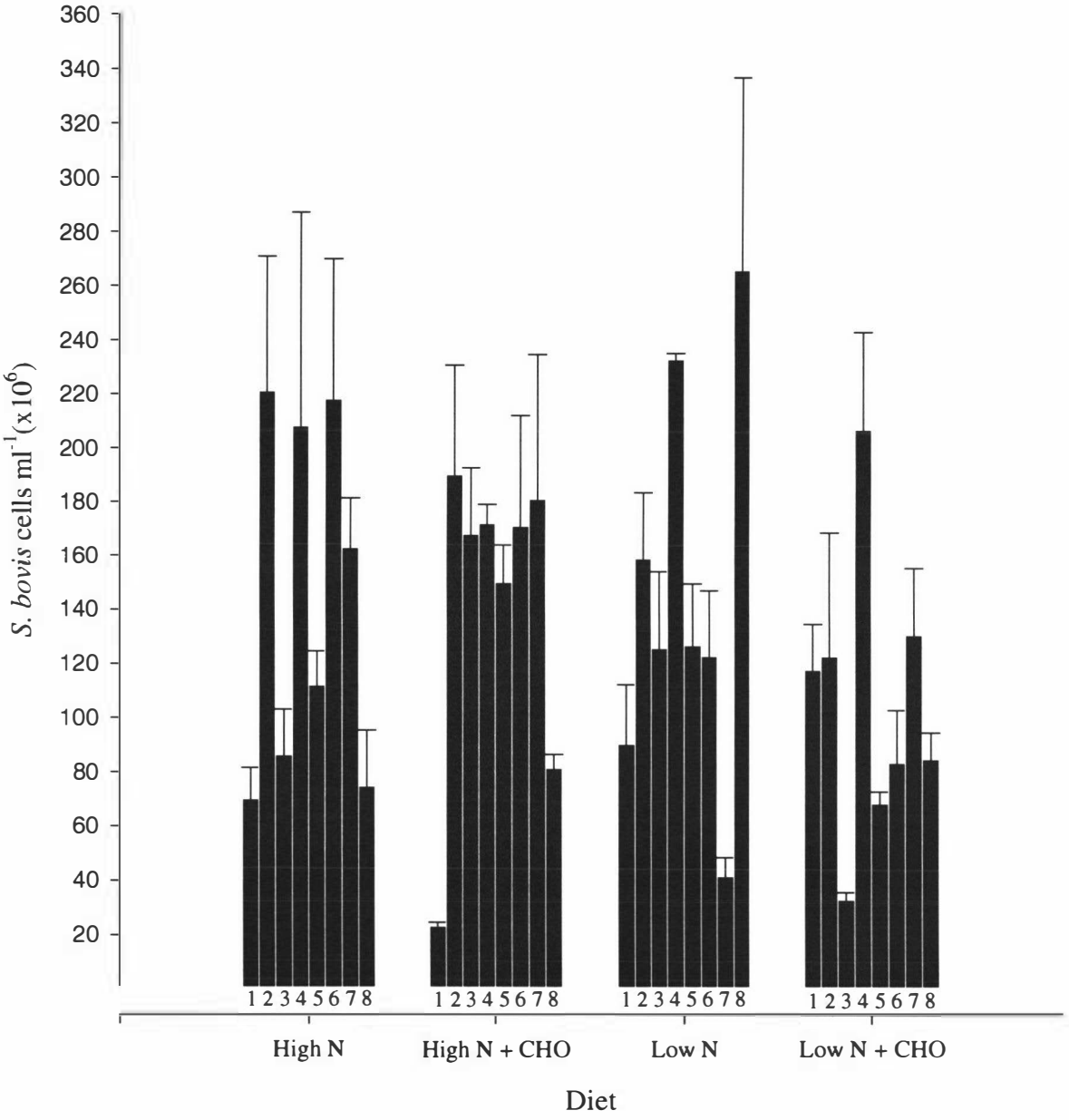

50
40
30
20
10
C. proteoclasticum cells ml-1 (x 10^6)
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
High N
High N +CHO
Low N
Low N+CHO
Diet

Figure 4.7. Populations of streptococci in dairy cows under four different feeding regimes.

Numbers 1 through 8 represent cows numbered 709, 710, 727, 788, 1758, 8702, 9754 and 9775 respectively. Results are the means of triplicate determinations and error bars represent standard error of the mean. The diets were fed in rotation and rumen samples were taken during each feeding regime.


360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
S. bovis cells ml-1 (x10^6)
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
High N
High N + CHO
Low N
Low N + CHO
Diet

4.3 Discussion

In order to determine the most efficient method of DNA extraction, three protocols were tested. Physical disruption by bead beating followed by phenol chloroform extractions was the most efficient method of extracting DNA. This method also had the advantage of assaying both the solid and liquid phases of rumen contents. The mechanical motion of bead beating displaces bacteria adhered to plant material, and allows sampling of the entire rumen ecosystem. This was important as the microbiota are more abundant in the total digesta than the liquid phase (Hungate, 1966), and previous studies on rumen populations have only sampled the liquid phase of rumen contents (Stahl *et al*, 1988; Briesacher *et al*, 1992; May *et al*, 1993).

Standard curves were constructed to relate the log ratio of target intensity/internal control intensity to log number of cells added. The standard curves were generated by coamplifying a serial dilution of DNA extracted from a known number of cells with a constant amount of internal control. The standard curves cover a wide range of cell numbers, 10^3-10^4 in the case of *C. proteoclasticum* and 2.5 x 10^2- 5 x 10^4 for the 1 x 10^5 dilution and 5 x 10^1 -1 x 10^4 for the 1 x 10^6 dilution of the *S. bovis* control. Two standard curves were needed for *S. bovis* quantitation, as some rumen samples did not amplify with the 1 x 10^5 dilution of the internal control. Constructing the standard curves using a serial dilution of DNA extracted from a known number of cells accounts for rRNA gene copy number which can range from 2 to 10 copies depending on the bacterium (Amikam *et al*, 1984; Jarvis *et al*, 1988). Standard curves also account for the small differences in amplification efficiencies observed between the internal control and the target DNA.

The sensitivity of competitive PCR was also determined in the same manner as internal control construction, where a serial dilution of DNA extracted from a known number of cells was coamplified with decreasing amounts of internal control, until no coamplification occured. In theory PCR is sensitive enough to detect a single copy of the target sequence (van Kuppeveld *et al*, 1992). However when the internal control is added to the PCR reaction the target DNA and internal control are competing for reagents and enzyme, therefore a decrease in sensitivity would be expected.

In the experiments a detection limit of DNA equivalent to 25 *C. proteoclasticum* cells (or 125 fg DNA) in a reaction was achieved when coamplified with a 2 x 10^6 dilution of the internal control. 25 cells was also the detection limit for *S. bovis*, but coamplified with a 5 x 10^6 dilution of the internal control. However, DNA extracted from rumen samples needed to be diluted 100 fold in order for reproducible amplification to occur, and so in practice a detection limit of 2.5 x 10^3 cells is the greatest sensitivity achieved. The exact nature of the inhibitory substance is unknown, but high $A_{260/280}$ readings indicate that it is not proteinaous in nature. It could possibly be a water soluble polysaccharide, or polyphenolic compound similar to that observed by Leser (1995) which interfered with PCR reactions.

The results from the testing of cPCR sensitivity compare favourably with those of previous investigations. Leser (1995) obtained a detection limit of 40 *Pseudomonas* cells using ethidium bromide stained gels visualised under UV for quantitation of cPCR products. A 10-fold improvement of sensitivity was achieved by transferring the DNA from the gel to membrane and hybridising with a specific probe although this increased the detection time. Kobayashi *et al.* (1998) also obtained similar results using cPCR to quantitate *F. succinogenes, E. ruminantium B. fibrisolvens* and *R. albus*, where a detection limit equivalent to between 100 and 1000 cells per PCR reaction was found. Quantitative PCR using a light-cycler based PCR system, instead of a cPCR system, also produced similar sensitivity results, with a detection system for *Listeria interrogans* able to detect between 100 and 400 cell copies per PCR reaction (Woo *et al.*, 1997a, 1997b).

The increased sensitivity of the PCR-based tests is an important improvement on the detection limit of conventional gene probing techniques used in rumen microbiology. The first study to use 16S rRNA probing in the rumen targeted *Fibrobacter* species (Stahl *et al.*, 1988). Three *Fibrobacter*, one *L. multiparus* and a universal probe that bound to all known 16S rRNA sequences were designed. These probes were used to study the effect of monensin on these bacteria *in vivo*. It was found that the technique was only sensitive enough to detect a specific organism if it comprised more than 0.01% of the total 16S rRNA (Stahl *et al.*, 1988). Gene probes targeting chromosomal genes following the fate of *Prevotella ruminicola* strain B_14 reintroduced to the rumen were only sensitive enough to detect a population of 2 x 10^7 cells ml^{-1} (Attwood et al., 1988).

In vitro analysis of fibrolytic rumen bacteria by Odenyo *et al.* (1994a; 1994b) demonstrated how 16S rRNA probing could be used to follow the populations of bacteria in co-culture. 16S rRNA probes were developed for *R. albus* and *R. flavefaciens*, and were used in conjunction with a *F. succinogenes* probe from previous studies (Stahl *et al.*, 1988). Co-culture experiments showed that *Ruminococcus albus* 8 out competed *R. flavefaciens* FD-1 when grown on cellobiose, while the growth of *Fibrobacter succinogenes* S85 was unaffected (Odenyo *et al.*, 1994a). Similar results were found when populations were grown on alkaline peroxide treated straw and cellulose (Odenyo *et al.*, 1994b). Krause and Russell (1996) designed 16S rRNA probes for *C. aminophilum, C. sticklandii* and *P. anaerobius* and followed the populations *in vivo* and in batch culture upon the addition of monensin. Each of these bacteria comprised approximately 1% of the total 16S rRNA population. On the addition of monensin the populations of *P. anaerobius* and *C. sticklandii* became undetectable, while *C. aminophilum* populations were unaffected (Krause and Russell, 1996). In these experiments bacterial abundance was expressed as a percentage of the total rRNA detected. While the method was able to follow individual bacterial species in defined co-cultures, it may not be sensitive enough to detect single strains from the rumen where background microbial populations are much larger. Indeed, in their study of *B. fibrisolvens* strains, Forster *et al.* (1997) could not detect individual strains in rumen contents using group-specific 16S rRNA probes. Of the three specific *B. fibrisolvens* probes designed, only probe 156 circumscribing 15 *B. fibrisolvens* isolates from White-tailed deer, hybridised to rumen contents from any of the animals tested. A detection limit for the 156 probe was determined by adding a known number of *B. fibrisolvens* cells to rumen fluid and extracting RNA. If 1000 ng of RNA was blotted onto the membrane the detection limit was 1×10^4 cells but if 100 ng of RNA was added the sensitivity dropped to 1×10^6 cells.

A drawback of the sensitivity of cPCR is that it can amplify the DNA from dead cells. While nucleic acids from dead cells are turned over rapidly in the rumen (McAllan and Smith, 1972), residual DNA from dead organisms may influence enumeration and cause over estimation of bacterial populations. Also, by targeting rDNA the cPCR technique determines bacterial numbers whereas probes targeting rRNA more accurately reflect the metabolic status of a population.

The effectiveness of competitive PCR was demonstrated by the *in vitro* assay of rumen contents. The correlation between the number of *C. proteoclasticum* cells added and *C. proteoclasticum* cells detected was good, though at higher numbers of *C. proteoclasticum* cells added there was a slight overestimation of population, possibly due to the resident *C. proteoclasticum* population in the rumen contents or errors in bacterial counting. In the *in vivo* detection experiments rumen samples collected from dairy cows fed diets differing in nitrogen and carbohydrate content were assayed for *C. proteoclasticum* and *S. bovis* populations using the competitive PCR technique. The populations of *C. proteoclasticum* ranged from 2.01 x 10^6 cells ml^{-1} on the high nitrogen diet to 3.12 x 10^7 cells ml^{-1} on the high nitrogen and carbohydrate supplemented diet. The populations of *C. proteoclasticum* determined by cPCR are in agreement with the original isolation of this organism from rumen contents. In this study changes in nitrogen or carbohydrate concentration produced no significant effect on the population of *C. proteoclasticum*, which is surprising, as this organism is highly proteolytic. However, increases in nitrogen in the diet may affect the regulation of proteinase genes within the organisms and would not necessarily be reflected in its growth. This has been observed before in *B. fibrisolvens* strains where changes in the nitrogen source in the media affected the proteolytic activity and this was not correlated to growth (Cotta and Hespell, 1986). Growth of *B. fibrisolvens* 49 on media containing casamino acids was not as high as growth on media containing casein or trypticase, but cells growing on the media containing casamino acids had the highest proteolytic activity. The amount of nitrogen in the media also affected proteolytic activity. Growth of *B. fibrisolvens* 49 on media containing 2.0% and 0.1% casamino acids was the same for the two cultures, but the proteolytic activity of the cells grown on 2.0% casamino acids was only 5% of the cells grown on 0.1% casamino acids.

The *S. bovis* population detected *in vivo* ranged from 1.7 x 10^7 to 1.3 x 10^8 cells ml^{-1}. The average population determined in this study of 1.3 x 10^8 ml^{-1} is in agreement with the original isolation from a 1 x 10^8 dilution of rumen contents. However this is higher than the average population of 10^7 ml^{-1} based on colony counts reported by Hungate (1966) from cattle on a wide variety of rations. In most animals the *S. bovis* population did not deviate as much as most other bacterial species. Enumerations using selective media also give similar results (Laukova, 1994). *S. bovis* populations have been observed to fluctuate daily, increasing for about 2 hours after the animal is fed and then

diminishing rapidly (Hungate, 1966). In this study the rumen samples used for cPCR enumeration were taken prior to the 4:00 p.m. feed. If rumen samples were taken soon after the 4:00 p.m. feeding the *S. bovis* population may have differed significantly, especially on the high carbohydrate diet which contained cornflour which is high in starch. However, these would likely have been only transient changes and would not reflect the overall effects of carbohydrate addition to the diet.

The populations of *C. proteoclasticum* and *S. bovis* appear to be stable in the rumen microbial ecosystem and do not fluctuate significantly with changes in carbohydrate or nitrogen in the diet. The finding that the populations of these organisms do not change significantly on a high nitrogen diet is surprising as these isolates were considered proteolytic, particularly *C. proteoclasticum.* The increased nitrogen in the diet however, could effect a response at the level of gene expression, rather than the increased growth of the organism.

Chapter 5 - General Discussion and Conclusions

The Polymerase Chain Reaction (PCR) is increasingly being applied in the field of molecular microbial ecology for the analysis of microbial populations directly from environmental samples. The main advantages of PCR are its speed and simplicity of analysis and its specificity and sensitivity of DNA detection. PCR has found use in the amplification of bacterial DNAs prior to restriction enzyme analysis, improving the sensitivity in dot-blot hybridisation (Steffan and Atlas, 1988), the cloning (Amann *et al.,* 1995) and sequencing (Edwards, *et al.*, 1989; Bottger, 1989; Weisburg *et al.*, 1990; Amann, *et al.,* 1995) of ribosomal RNA genes, denaturing gradient gel electrophoresis (DGGE) and competitive PCR (Leser, 1995; Leser *et al.,* 1995; Lee *et al.,* 1996; Kobayashi *et al.,* 1998). In this study, a competitive PCR technique has been developed for two proteolytic rumen bacteria and the usefulness of the technique for detecting these bacterial populations directly from rumen samples has been assessed.

The cPCR technique reported here targets the 16S rDNAs of proteolytic rumen bacteria. The first of these organisms, *C. proteoclasticum*, is an extremely active protein degrading bacterium isolated from a pasture-grazed cow which shares many characteristics with *B. fibrisolvens.* The major differences between the species are in the fermentation of glycerol, glycogen and starch (Attwood and Reilly, 1995), and proteolytic activity (Attwood and Reilly, 1996). The current species description of *B. fibrisolvens* encompasses a diverse group of organisms, with wide ranging proteolytic and cellulolytic characteristics (Bryant and Small, 1956; Cotta and Hespell, 1986, Fulghum and Moore, 1963). *B. fibrisolvens* has been found to be the predominant proteolytic organism isolated from some animals (Fulghum and Moore, 1963), and some strains have very high proteolytic activities (Cotta and Hespell, 1986, Attwood and Reilly, 1996). The basis for classification of strains in the genus *Butyrivibrio*has been as gram negative, obligately anaerobic, curved rods, which produce butyrate as the main product of carbohydrate fermentation, and do not produce spores (Bryant, 1986). This broad description has meant a diverse assemblage of organisms has been described within the genus. Recently 16S rDNA analysis and DNA/DNA homology has divided *B. fibrisolvens* into at least two major groups (Mannarelli, 1988; Mannarelli *et al.,* 1990; Forster *et al.*, 1996; Willems *et al.*, 1996). *C. proteoclasticum* is closely related to *B.*

fibrisolvens strains NCDO 2435, 2434, 2222, 2432 and 2398 and this group seems to form a cluster with 96.9 to 99.5% similarity in their 16S rDNA sequences. However this group is only distantly related to the *B. fibrisolvens* type strain, NCDO 2221^T, with sequence similarities ranging from 93.1 to 94.1%. Therefore the *C. proteoclasticum* group seems to constitute a cohesive assemblage of strains which probably represent a new species.

At the beginning of this thesis, the *C. proteoclasticum* cPCR primer was designed by aligning and comparing 16S sequences from closely related organisms and identifying regions which discriminated between bacterial species. The Ribosomal RNA Database Project (RDP), as the official repository for ribosomal RNA sequences, was used to obtain sequence information for these comparisons. Based on these comparisons the primer was specific for *C. proteoclasticum* alone and this was confirmed using the RDP PROBE-CHECK facility (Olsen *et al.*, 1993). However, not all rRNA sequences have been deposited at the RDP and it was subsequently discovered that four of the closely related *B. fibrisolvens* strains (NCDO strains 2435, 2432, 2222 and 2398) mentioned above also shared the *C. proteoclasticum* priming site.

The three *S. bovis* isolates were confirmed as *S. bovis* strains by 16S rDNA analysis, and the two isolated from New Zealand ruminants had virtually identical 16S rRNA sequence (99.9%). This tight clustering of *S. bovis* strains was also observed by Nelms *et al.* (1995). DNA/DNA homology and 16S rDNA information identified two distinct homology groups among ruminal *S. bovis* strains (Nelms *et al.* 1995). Isolates from within each of these groups were closely related to each other, but not closely related to members of the other group (Nelms *et al.* 1995). The design of a *S. bovis*-specific primer was confounded by the incompleteness of the RDP sequences such that the *S. bovis*-specific cPCR primer site also appears in *S. rattus*, *S. mutans*, *S. milleri*, *S. intestinalis*, *S. equinus*, *S. caprinus*. *S. constellatus*, *S. alactolyticus* and *S. macedonicus*. Unlike the *C. proteoclasticum-B. fibrisolvens* strains, these *Streptococcus* species do not form a phylogenetically cohesive cluster. However, in terms of the rumen microbial environment, the primer circumscribes the ruminal *Streptococcus* species (ie. *S. bovis* and *S. caprinus)* and as such is useful as a ruminal *Streptococcus* genus probe.

As can be seen from the examples of *C. proteoclasticum* and *S. bovis*, targeting the 16S rRNA gene allows the construction of probes to different taxonomic levels. This is due to the rRNA molecule containing distinct regions of both conserved and variable sequence that can be targeted to design probes to higher or lower taxonomic groupings respectively. This is a powerful capability as it means that probes can be designed in a phylogenetically "nested" fashion allowing the investigation of bacterial communities at each taxonomic level (Amann *et al.,* 1995). The increasing number of 16S rRNA sequences that are being reported, and often now required, in the description of new bacterial species also means there is also an ever increasing database from which to design and verify probes.

The cPCR technique was originally developed as a tool for quantitation of mRNA from the HIV-1 virus (Zachar *et al.*, 1993) where one of its main advantages was its sensitivity, being able to detect low levels of mRNA. Zachar *et al.* (1993) did not quantitate the absolute detection limit for their cPCR system, however Piatak *et al.* (1993) were able to detect as few as 100 copies of HIV-1 mRNA per reaction using a similar competitive RT-PCR technique. The sensitivity of the cPCR assay for both *C. proteoclasticum* and *S. bovis* detection was 25 cells per reaction. This was determined by extracting DNA from a known number of either *C. proteoclasticum* or *S. bovis* cells and performing cPCR assays with decreasing amounts of target and internal control DNAs until no coamplification was detected. This is an absolute detection limit as it does not take into account the dilution of DNA extracted from rumen samples required to allow the coamplification to operate. This 100-fold dilution places the practical sensitivity of the technique at 2.5×10^3 cells per reaction. cPCR appeared to be less sensitive than PCR conducted without an internal control. Theoretically the assays should have equivalent sensitivities but this is technically difficult to achieve. At the theoretical point of greatest sensitivity, coamplification requires exactly one molecule each of target and internal control DNA, which in practical terms is almost impossible to achieve. Therefore the practical sensitivity limit is determined by the point where both target and internal control DNAs are at a level which allow coamplification to occur. The sensitivity of the cPCR assay compared favourably with results found by other groups using PCR detection methods. cPCR detection limits found ranged between 40 and 1000 cells per PCR reaction (Leser, 1995; Kobayashi *et al.,* 1998) and

quantitative PCR using a rapid-time PCR detection method ranged from 100-400 copies per PCR reaction (Woo *et al.*, 1997a; 1997b).

Competitive PCR has proven to be a rapid, reliable and accurate method for detection of bacteria from rumen samples. The development of the internal control and construction of standard curves are the most time-consuming phases of the process. In this study internal control development relied on the presence of convenient restriction endonuclease sites within the target 16S gene. Such sites may not be available in all cases and therefore one may need to revert to other methods for generating an internal DNA control. However, once the internal control is made enumerating rumen samples using cPCR takes little time. Similarly, the construction of standard curves with the appropriate dilution of internal control takes time. Indeed, it may require the use of two sets of standard curves to cover the range of bacterial numbers encountered as was the case with the detection of *S. bovis*. Therefore cPCR combines the sensitivity of the PCR reaction with the specificity of designing probes to the variable regions of 16S rRNA genes. This technique also overcomes the reluctance to recognise PCR as a quantitative technique as variation between reactions is standardised by the inclusion of the internal control.

The finding that *C. proteoclasticum* and closely related *B fibrisolvens* strains are present in the rumen between 2.1 x 10^6 and 3.1 x 10^7 ml^{-1} and that their population does not differ significantly on different diets suggests that these organisms are stable residents of the ecosystem. *B. fibrisolvens* can grow with intact protein as the sole nitrogen source (Wallace and Brammall, 1985), and is probably involved in the primary hydrolysis of feed protein and digestion of carbohydrates. *C. proteoclasticum* probably fulfils a similar role in the rumen as it shares many characteristics with *B. fibrisolvens* (Attwood and Reilly, 1995; Attwood and Reilly 1996; Attwood *et al.*, 1996). *C. proteoclasticum* has a very high chymotrypsin-like serine-type proteinase activity (Attwood and Reilly, 1996). This observation is in keeping with previous findings where strains of *B. fibrisolvens* as well as *R. amylophilus* and *Fusobacterium* spp. also had high serine proteolytic activity (Wallace and Brammall, 1985). The high serine proteinase activity of these bacteria was not reflected in the activity of mixed rumen bacteria, which had a predominantly cysteine type proteinase. Detailed studies of *B. fibrisolvens* strain H17c revealed that all nine extracellular proteinases found were

serine proteinases (Strydom *et al.*, 1986), which is consistent with the predominantly serine type proteinase activity associated with *B. fibrisolvens* (Attwood and Reilly, 1996: Wallace and Brammall, 1985). Of the proteolytic bacteria isolated from New Zealand cattle, *C. proteoclasticum* had the highest casein hydrolysing ability (Attwood and Reilly, 1995; Attwood and Reilly, 1996), and was isolated from a 1 x 10^8 dilution of rumen contents. It would be interesting to compare the proteolytic activity of the five closely related *B. fibrisolvens* strains to see whether the populations measured by the B316 830 primer reflect the high proteolytic activity possessed by *C. proteoclasticum*

B. fibrisolvens has been found to be one of the predominant bacteria isolated from the rumen, consistently being cultured from 10^8 dilutions of rumen fluid (Bryant and Small, 1956; Fulghum and Moore, 1963; Hungate, 1950). However identification of isolates in these enumerations were based on cell morphology and it is now accepted that the morphology of *B. fibrisolvens* encompasses many species and probably at least two genera (Mannarelli, 1988; Mannarelli *et al.*, 1990; Forster *et al.*, 1996; Willems *et al.*, 1996). Previous attempts to enumerate specific strains of *B. fibrisolvens* using molecular techniques were unsuccessful due to the insensitivity of 16S rRNA probing (Forster *et al.*, 1997).

S. bovis is one of the most commonly isolated rumen bacteria, as it does not require a low oxidation-reduction potential, and grows very rapidly (Perry and Briggs, 1955) making culturing less complicated. While it does not have high proteinase activity, it does have a very high leucine aminopeptidase activity and probably contributes very actively to exopeptidase activity in the rumen (Wallace and Brammall, 1985). In New Zealand cattle *S. bovis* isolates comprised only 32% of the total casein hydrolysing ability, even though they comprised 61% of the 43 characterised proteolytic isolates (Attwood and Reilly, 1995). This low proteolytic, but high leucine aminopeptidase activity may explain the organism's ability to take up amino acids, but not peptides (Ling and Armstead, 1995).

The results from this study indicate that cPCR has a role in future molecular microbial investigations in the rumen ecosystem. Its combined specificity and sensitivity are well suited to such a complex and densely populated environment. Future work will extend the use of the cPCR technique for analysis of other proteolytic bacteria in New Zealand ruminants. The application cPCR may extend beyond the rumen, as any microbial

community can be interrogated using specific primers and internal controls designed for any desired taxonomic level. The ability to clone 16S rDNA sequences allows a bacterium to be classified, design primers for the organism and enumerate it using cPCR without the organism ever having been cultured. The design of truly universal PCR primers and an internal control for the enumeration of total bacterial population would also circumvent the need for enumeration by culturing. This approach would also allow the expression of a bacterial population as a proportion of the total population. The recent development of rapid-time PCR using light-cycling and fluorescent probe technology may be the next step in the enumeration of microbial populations from the environment. Fluorescence resonance energy transfer (FRET) probes which have been used to detect and quantitate bacterial populations (Woo *et al.*, 1997a; 1997b) may be amenable to use in cPCR reactions so that internal control DNAs could be monitored at the same time as target DNAs. Finally, the enumeration of *C. proteoclasticum* and *S. bovis* populations in this study has found relatively stable populations despite changes in the level of nitrogen and carbohydrate in the diet. Responses to these dietary changes might therefore be expected at the level of proteinase expression. Future work on bacterial proteinase genes and their expression under different nitrogen conditions will hopefully provide a better understanding of the contribution of specific bacterial populations to rumen proteolysis.

APPENDIX A: MEDIA

Anaerobic media

Complete carbohydrate (Leedle and Hespel, 1980)[a],

Ingredient	per 100 ml
Carbohydrate[b]	0.45 g
Trypticase (Difco)	0.2 g
Yeast extract	0.05 g
Minerals I[c]	4.0 ml
Minerals II[c]	4.0 ml
Haemin[d] (0.01%)	0.1 ml
Volatile fatty acid solution[e]	1.0 ml
Resazurin (0.1%)	0.1 ml
Rumen Fluid	16.0 ml
Na_2S/L-cysteine hydrochloride solution (2.5%/2.5%),	1.0 ml
Na_2CO_3	0.4 g

[a] All components of the media except Na_2CO_3 and the cysteine sulfide were dissolved in the appropriate volume of water and the pH was adjusted to 6.5 with 1 M HCl. The media was boiled until the resasurin changed from red to colourless. The media was cooled under CO_2 gas flow before adding the Na_2CO_3 and dispensed into either 15ml Hungate tubes or serum bottles flushed with CO_2. The containers were then sealed and autoclaved. Na_2S/L-cysteine hydrochloride solution was added immediately before inoculation .

[b] Carbohydrate: cellulose, cellobiose, glucose, maltose, pectin, soluble starch, xylan and xylose 0.05% each, plus glycerol 0.05% (v/v)

[c] MINERALS I

K_2HPO_4	0.6

MINERALS II

KH_2PO_4	0.6
$(NH_4)_2SO_4$	0.6
NaCl	1.2
$MgSO_4.7H_2O$	0.255
$CaCl_2.2H_2O$	0.169

[d] HAEMIN SOLUTION

10mg of Haemin dissolved in 50 ml of ethanol plus 50 ml of 0.05 M NaOH

[e] VOLATILE FATTY ACID SOLUTION

Acetic Acid,	17 (v/v)
Propionic	6 (v/v)
Butyric	4 (v/v)
iso-butyric	1 (v/v)
n-valeric	1 (v/v)
DL-α-methylbutyric	1 (v/v)

Ingredients added and the pH adjusted to 7.5 with NaOH. The volume was adjusted to 100mls with distilled water.

Aerobic Media.

Luria-Bertani (LB) Broth. (Miller, 1972) per litre

NaCl,	5.0g
Bacto Tryptone (Difco)	10g
Yeast Extract (Difco)	5.0 g

The pH was adjusted to 7.0 with 1 M NaOH before sterilization.

SOB Medium	per litre
Bacto Tryptone (Difco),	20g
Yeast extract,	5.0g
NaCl	0.5g
250 mM KCl,	10 ml
2M $MgCl_2$	5.0 ml

The solutes were dissolved in 950 ml water and the pH was adjusted to 7.0 with 5M NaOH. The volume was adjusted to 1 litre and sterilized. The sterile Mg_2Cl_2 was added just before use of the media. Solid SOB agar contained 1.5% agar.

SOC Medium is the same as SOB medium, except after autoclaving, 2 ml of sterile 1M glucose solution is added.

APPENDIX B: BUFFERS AND SOLUTIONS

Gel Loading Buffer	per 100ml
Bromophenol Blue	0.25g
Xylene Cyanol	0.25g
Ficol (type 400 in H_2O)	25g

Reagents are combined and can be stored at room temperature.

IPTG

1g of IPTG (Isopropylthio-β-D-Galactoside) (Boehringer Mannheim, Germany) was dissolved up in 5 ml of distilled water and filter sterilized before freezing 500 µl aliquots, which were thawed immediately before use.

Phenol

Ultra pure phenol (Gibco BRL, Life Technologies) was obtained as redistilled solid bottled under nitrogen, which was then stored at -20°C until use. To equilibrate the phenol, it was removed from the freezer and allowed to reach room temperature, before melting at 65°C. An equal volume of distilled water was added to the melted phenol, and mixed to combine the two phases. The emulsion was left overnight for the phases to separate out before the acid phenol was aliquoted out for storage at 4°C. To raise the pH of the phenol, the aqueous phase was removed, and an equal volume of 0.5M Tris.HCl pH 8.0 and 0.1% (w/v) hydroxyquinoline was added and stirred overnight. Once taken off the stirrer the phases were allowed to separate, the aqueous phase removed and the pH of the phenol measured. This process was repeated until the pH of

the phenol was above 7.8. Once the pH was adjusted correctly then the phenol was covered by 0.1 M Tris.HCl pH 8.0 and stored in dark bottle at 4°C for a month.

P1 (Resuspension Buffer)

Tris.HCl (Sigma)	50mM
EDTA	10mM
RNase A	100µg ml^{-1}

The EDTA and Tris were combined and the pH adjusted to 8.0 with HCl. The solution was autoclaved and the RNase A added, before storage at 4°C.

P2 (Lysis Buffer)

Na OH	200mM
SDS	1% (W/V)

P3 (Neutralisation Buffer)

$KC_2H_3O_2$	3.0M

Th $KC_2H_3O_2$ was dissolved in water and the pH was adjusted to 5.5 with glacial acetic acid, and the solution sterilised by autoclaving, and stored at 4°C.

QC (Wash Buffer)

NaCl	1.0m
MOPS	50mM
Ethanol	15% (v/v)

The NaCl and MOPS were dissolved in distilled water, and the pH adjusted to 7.0, before autoclaving. Once the solution was cool, then the ethanol was added.

QBT (Equilibration Buffer)

NaCl	750mM
MOPS	50mM
Ethanol	15% (v/v)
Triton X-100	0.15% (v/v)

The NaCl and MOPS were dissolved in distilled water, and the pH adjusted to 7.0 and the solution autoclaved. The Triton X-100 and ethanol were then added once the solution had cooled.

QF (Elution Buffer)

NaCl	1.25M
Tris	50mM
Ethanol	15% (v/v)

The NaCl and Tris base were dissolved in distilled water, and the pH adjusted to 8.5 before autoclaving. The ethanol was added once the solution had cooled, and the pH checked for drift and readjusted.

Saline EDTA

0.15 M NaCl

0.1 M EDTA pH 8.0

TAE

(10x working concentration)	per 100ml
Tris base	4.84
Glacial acetic acid	1.12
0.5M EDTA (pH 8.0)	2.0

No adjustment of pH

TE

10 mM Tris.HCl	pH 8.0
1 mM EDTA	pH 8.0

TEG

50 mM Glucose

25 mM Tris.HCl (pH8.0)

10 mM EDTA (pH 8.0)

X-GAL (5-bromo,-4-chloro-3-indolyl-β-D-Galactoside) (Promega) was stored at -20°C in formaldehyde at a concentration of 50 mg ml^{-1}

Appendix C

16S rRNA sequences of proteolytic rumen bacteria isolated from New Zealand ruminants. Alignments of sequencing fragments are also shown.

C. proteoclasticum 16S rDNA sequence

```
   9  GAGTTTGATC CTGGCTCAGG ATGAACGCTG GCGGCGTGCC TAACACATGC
  59  AAGTCGAACG GAGATATAAC GCTGCAGAGA CTTCGGTCAA AGCTTGTTGT
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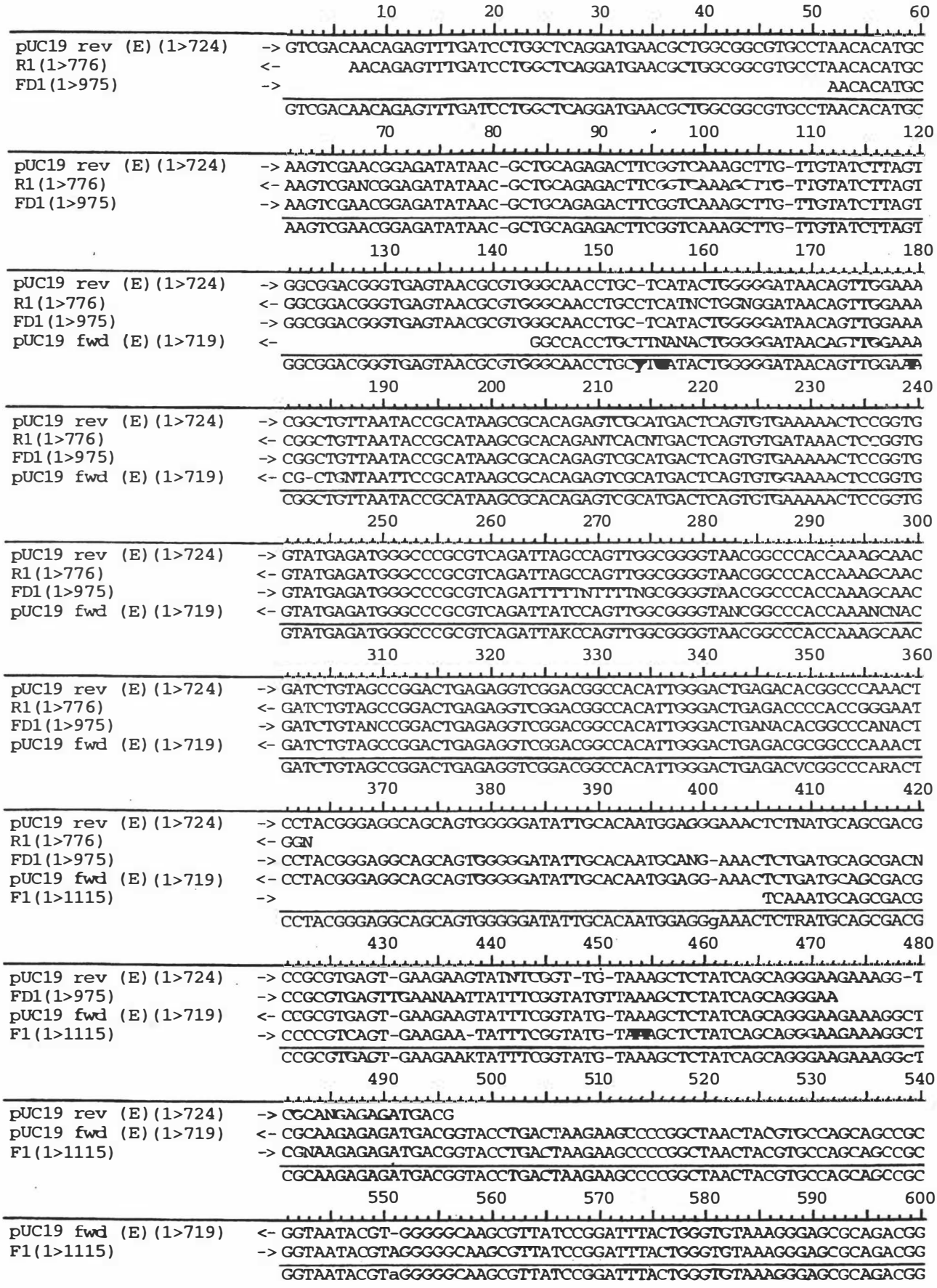
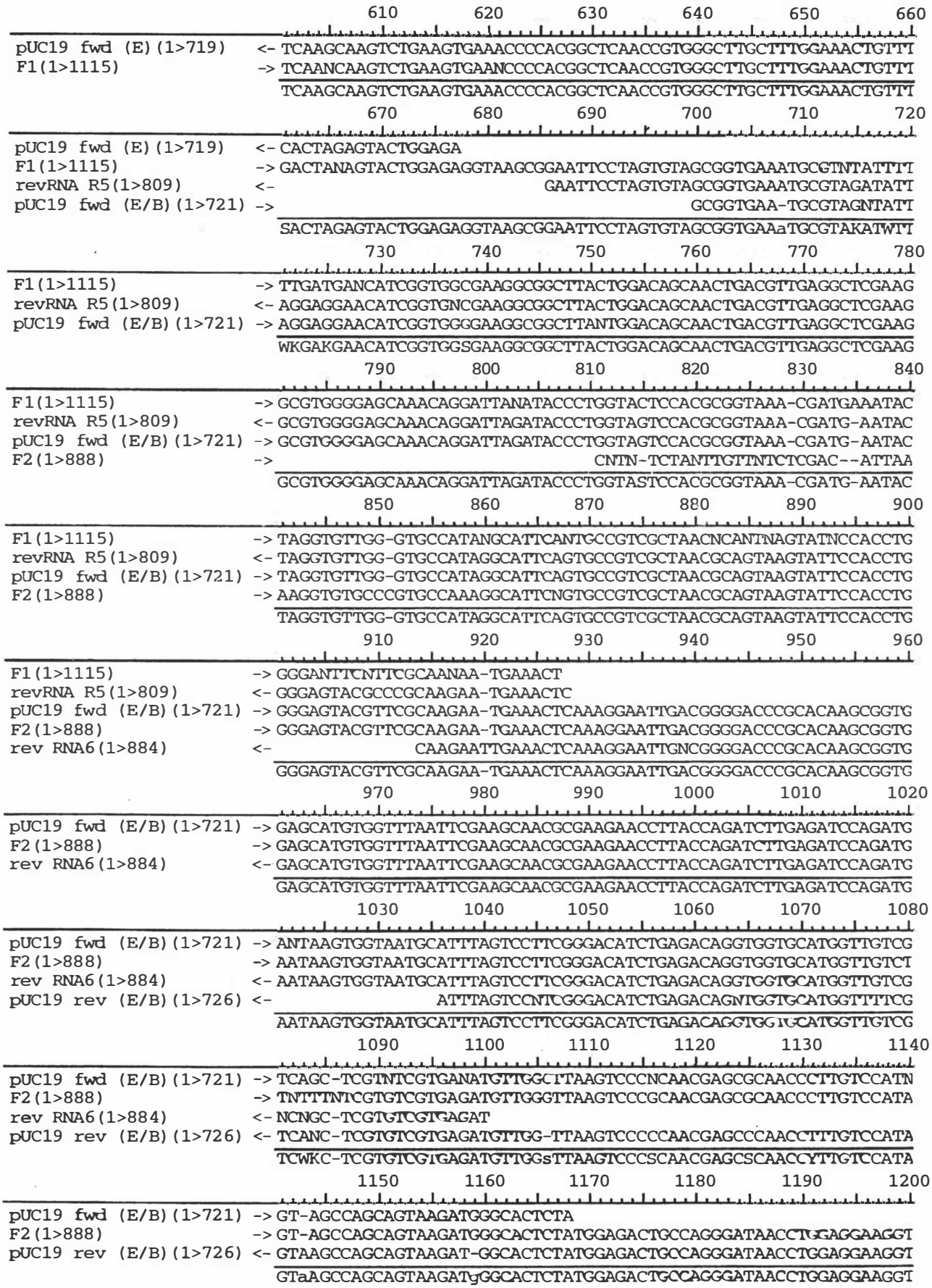
C. proteoclasticum sequence alignments

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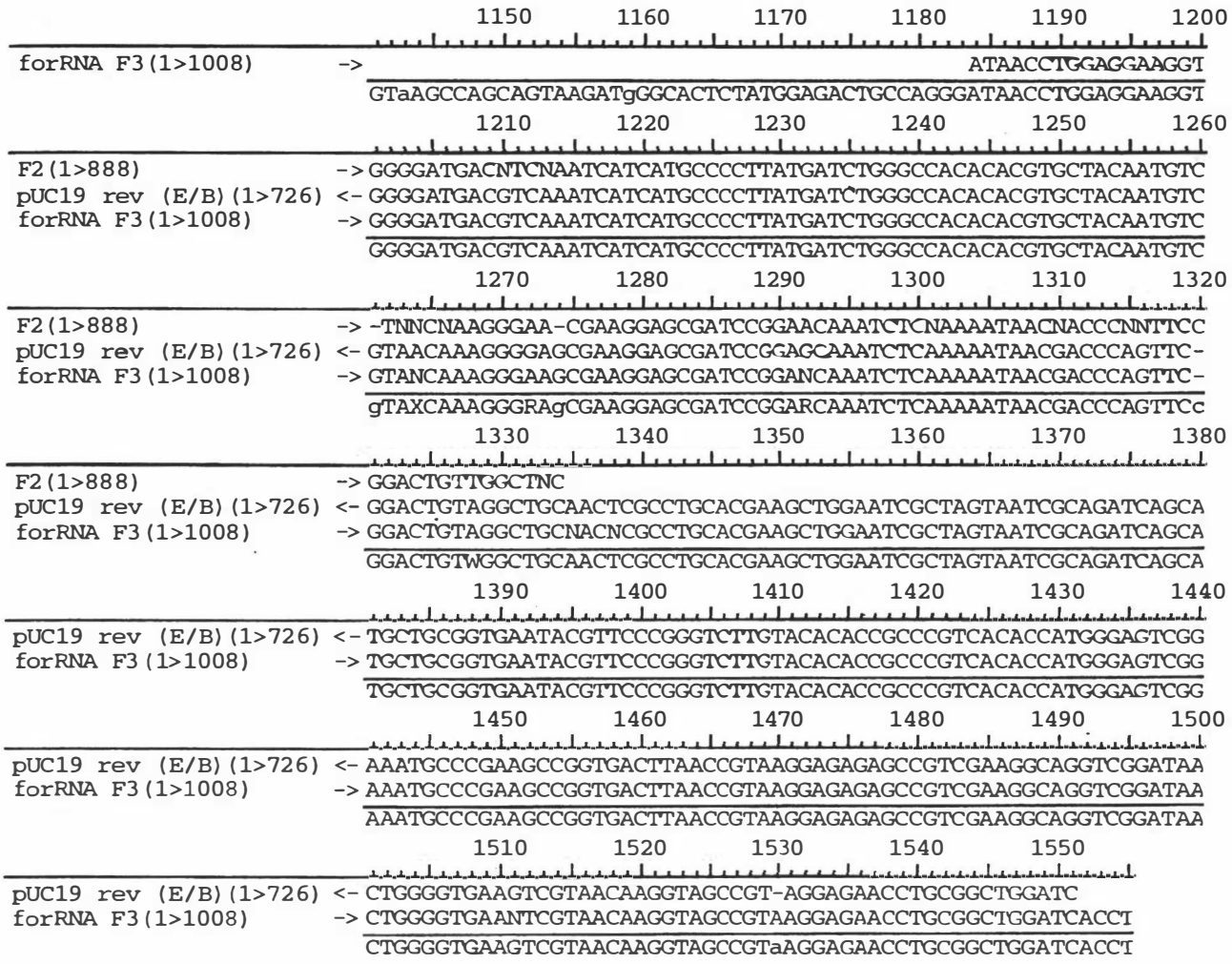
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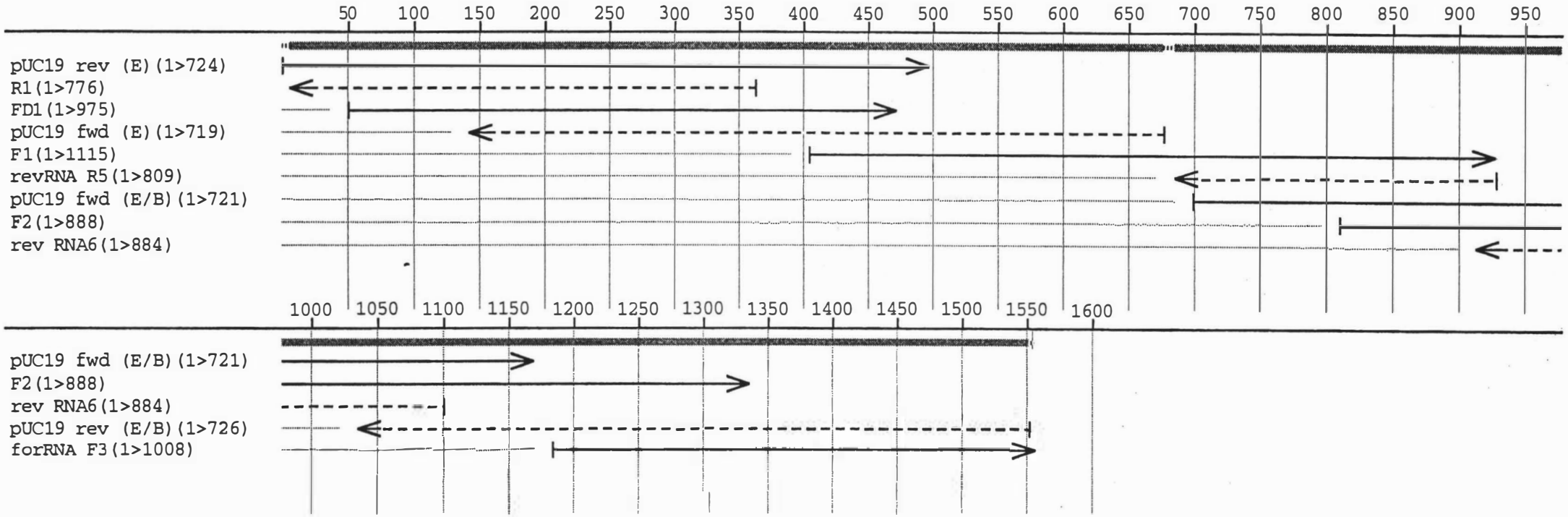
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F2(1>888)
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S. bovis NCBF 2476, 16S rRNA

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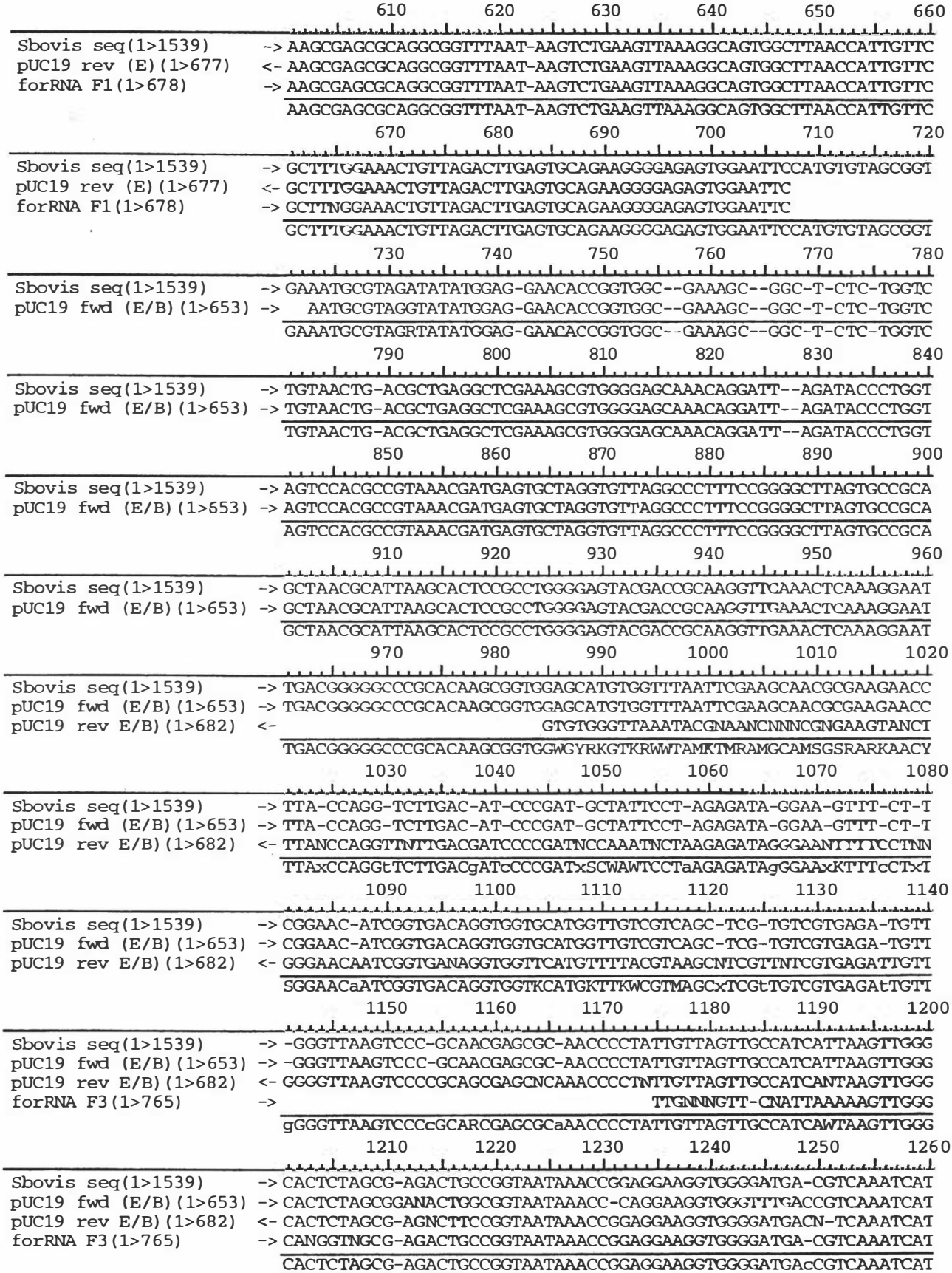
S. bovis NCFB 2476 sequence alignments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GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAAT
970 980 990 1000 1010 1020
Sbovis seq(1>1539) -> TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
pUC19 fwd (E/B)(1>653) -> TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
pUC19 rev E/B)(1>682) <- GTGTGGGTTAAATACGNAANCNNNCGNGAAGTANCT
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1030 1040 1050 1060 1070 1080
Sbovis seq(1>1539) -> TTA-CCAGG-TCTTGAC-AT-CCCGAT-GCTATTCCT-AGAGATA-GGAA-GTTT-CT-T
pUC19 fwd (E/B)(1>653) -> TTA-CCAGG-TCTTGAC-AT-CCCGAT-GCTATTCCT-AGAGATA-GGAA-GTTT-CT-T
pUC19 rev E/B)(1>682) <- TTANCCAGGTTNTTGACGATCCCCGATNCCAAATNCTAAGAGATAGGGAANTTTTCCTNN
TTAxCCAGGtTCTTGACgATcCCCGATxSCWAWTCCTaAGAGATAgGGAAxKTTTcCTxT
1090 1100 1110 1120 1130 1140
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pUC19 fwd (E/B)(1>653) -> CGGAAC-ATCGGTGACAGGTGGTGCATGGTTGTCGTCAGC-TCG-TGTCGTGAGA-TGTT
pUC19 rev E/B)(1>682) <- GGGAACAATCGGTGANAGGTGGTTCATGTTTTACGTAAGCNTCGTTNTCGTGAGATTGTT
SGGAACaATCGGTGACAGGTGGTKCATGKTTKWCGTMAGCxTCGtTGTCGTGAGAtTGTT
1150 1160 1170 1180 1190 1200
Sbovis seq(1>1539) -> -GGGTTAAGTCCC-GCAACGAGCGC-AACCCCTATTGTTAGTTGCCATCATTAAGTTGGG
pUC19 fwd (E/B)(1>653) -> -GGGTTAAGTCCC-GCAACGAGCGC-AACCCCTATTGTTAGTTGCCATCATTAAGTTGGG
pUC19 rev E/B)(1>682) <- GGGGTTAAGTCCCCGCAGCGAGCNCAAACCCCTNTTGTTAGTTGCCATCANTAAGTTGGG
forRNA F3(1>765) -> TTGNNNGTT-CNATTAAAAAGTTGGG
gGGGTTAAGTCCCcGCARCGAGCGCaAACCCCTATTGTTAGTTGCCATCAWTAAGTTGGG
1210 1220 1230 1240 1250 1260
Sbovis seq(1>1539) -> CACTCTAGCG-AGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGA-CGTCAAATCAT
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pUC19 rev E/B)(1>682) <- CACTCTAGCG-AGNCTTCCGGTAATAAACCGGAGGAAGGTGGGGATGACN-TCAAATCAT
forRNA F3(1>765) -> CANGGTNGCG-AGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGA-CGTCAAATCAT
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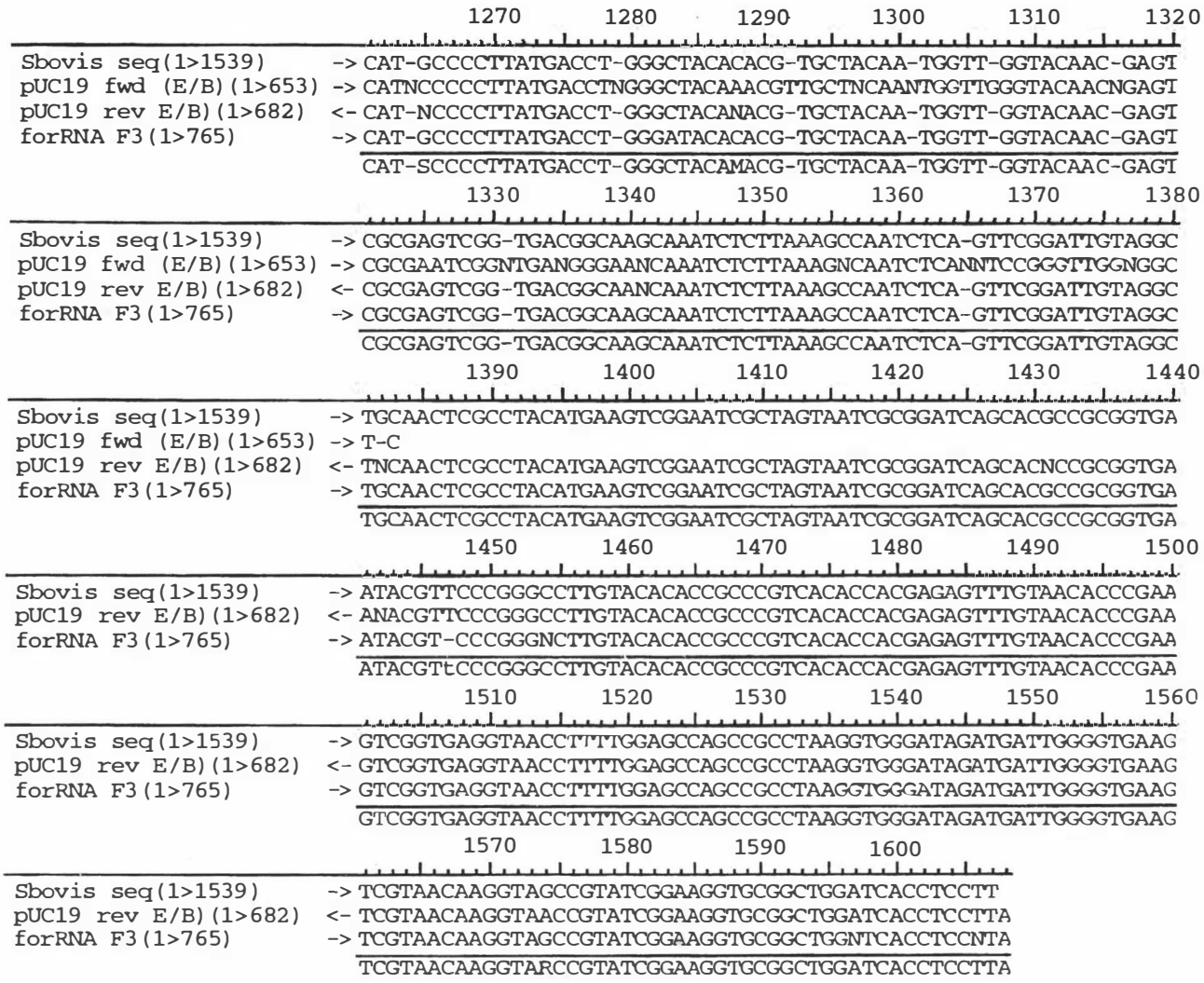
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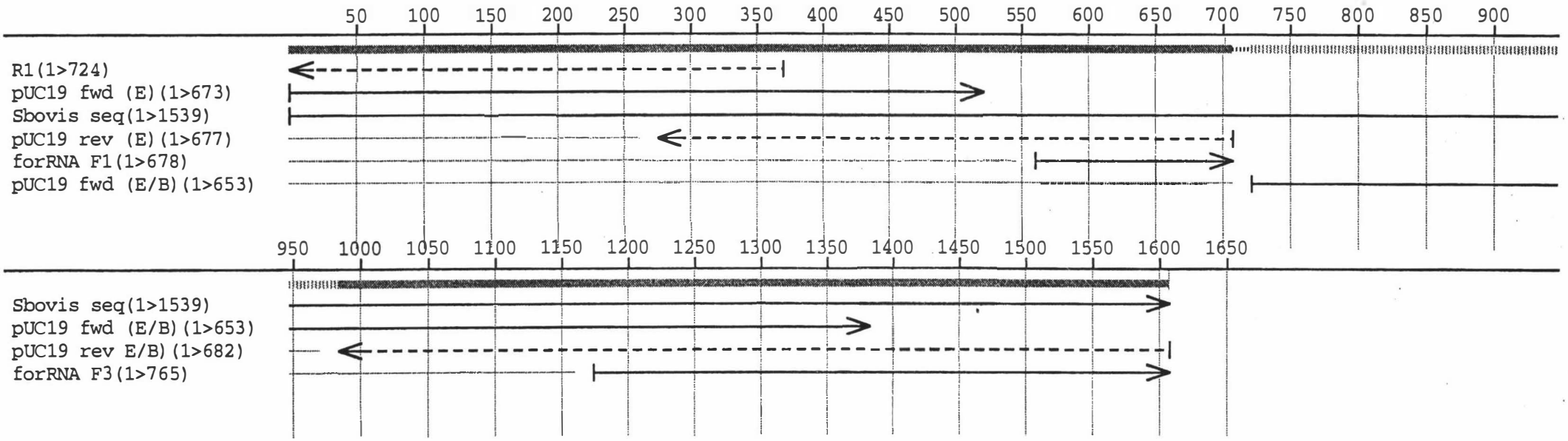
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pUC19 rev (E)(1>677)
forRNA F1(1>678)
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Sbovis seq(1>1539)
pUC19 fwd (E/B)(1>653)
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S. bovis, B315, 16S rRNA

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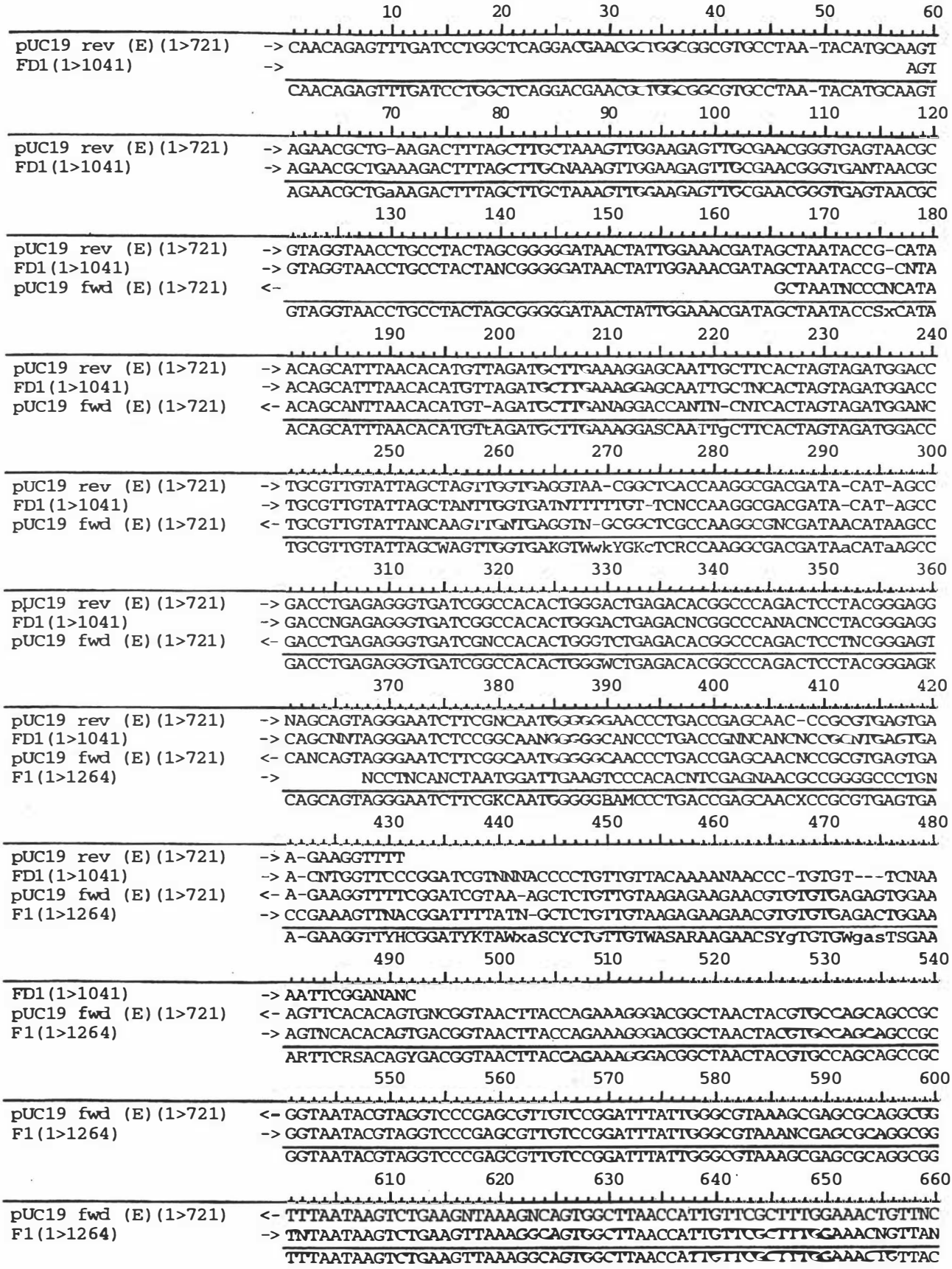
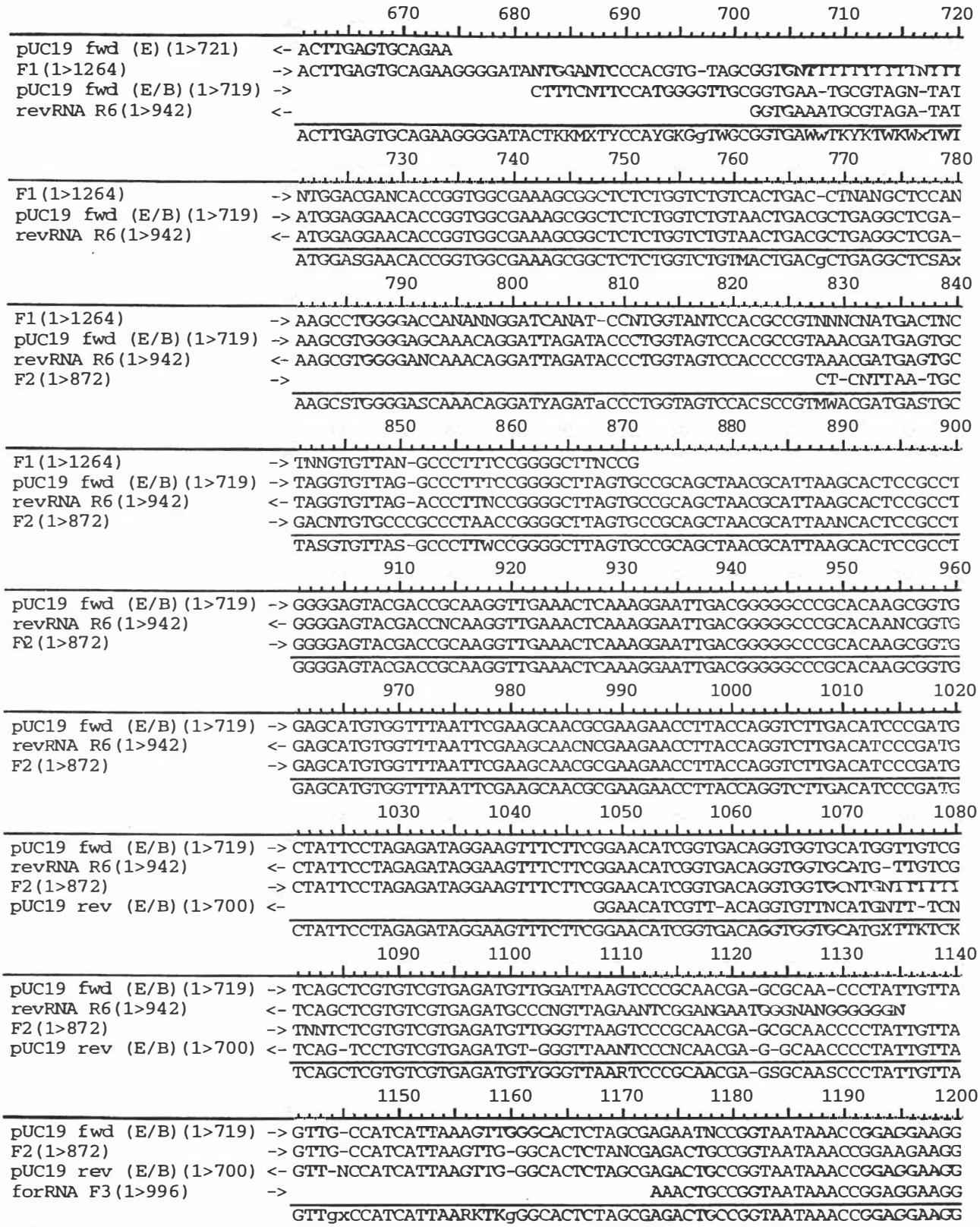
S. bovis B315 sequence alignments

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pUC19 fwd (E/B)(1>719) -> CTTTCNTTCCATGGGGTTGCGGTGAA-TGCGTAGN-TAT
revRNA R6(1>942) <- GGTGAAATGCGTAGA-TAT
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730 740 750 760 770 780
F1(1>1264) -> NTGGACGANCACCGGTGGCGAAAGCGGCTCTCTGGTCTGTCACTGAC-CTNANGCTCCAN
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revRNA R6(1>942) <- ATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGA-
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790 800 810 820 830 840
F1(1>1264) -> AAGCCTGGGGACCANANNGGATCANAT-CCNTGGTANTCCACGCCGTNNNCNATGACTNC
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revRNA R6(1>942) <- AAGCGTGGGGANCAAACAGGATTAGATACCCTGGTAGTCCACCCCGTAAACGATGAGTGC
F2(1>872) -> CT-CNTTAA-TGC
AAGCSTGGGGASCAAACAGGATYAGATaCCCTGGTAGTCCACSCCGTMWACGATGASTGC
850 860 870 880 890 900
F1(1>1264) -> TNNGTGTTAN-GCCCTTTCCGGGGCTTNCCG
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revRNA R6(1>942) <- TAGGTGTTAG-ACCCTTNCCGGGGCTTAGTGCCGCAGCTAACGCATTAAGCACTCCGCCT
F2(1>872) -> GACNTGTGCCCGCCCTAACCGGGGCTTAGTGCCGCAGCTAACGCATTAANCACTCCGCCT
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910 920 930 940 950 960
pUC19 fwd (E/B)(1>719) -> GGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
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F2(1>872) -> GGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
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revRNA R6(1>942) <- CTATTCCTAGAGATAGGAAGTTTCTTCGGAACATCGGTGACAGGTGGTGCATG-TTGTCG
F2(1>872) -> CTATTCCTAGAGATAGGAAGTTTCTTCGGAACATCGGTGACAGGTGGTGCNTGNTTTTTT
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revRNA R6(1>942) <- TCAGCTCGTGTCGTGAGATGCCCNGTTAGAANTCGGANGAATGGGNANGGGGGGN
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1150 1160 1170 1180 1190 1200
pUC19 fwd (E/B)(1>719) -> GTTG-CCATCATTAAAGTTGGGCACTCTAGCGAGAATNCCGGTAATAAACCGGAGGAAGG
F2(1>872) -> GTTG-CCATCATTAAGTTG-GGCACTCTANCGAGACTGCCGGTAATAAACCGGAAGAAGG
pUC19 rev (E/B)(1>700) <- GTT-NCCATCATTAAGTTG-GGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGG
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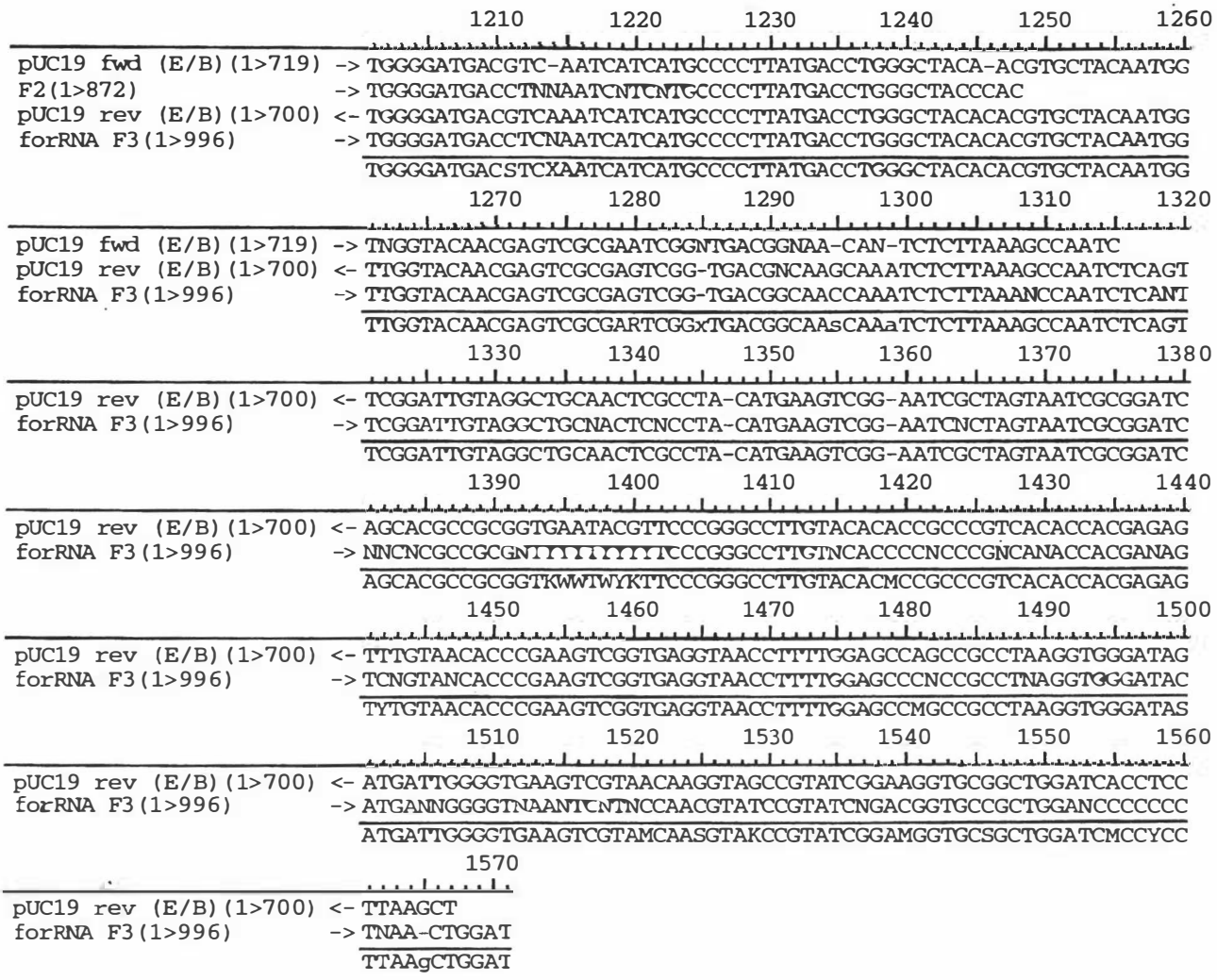
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1450 1460 1470 1480 1490 1500
pUC19 rev (E/B)(1>700) <- TTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGATAG
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1510 1520 1530 1540 1550 1560
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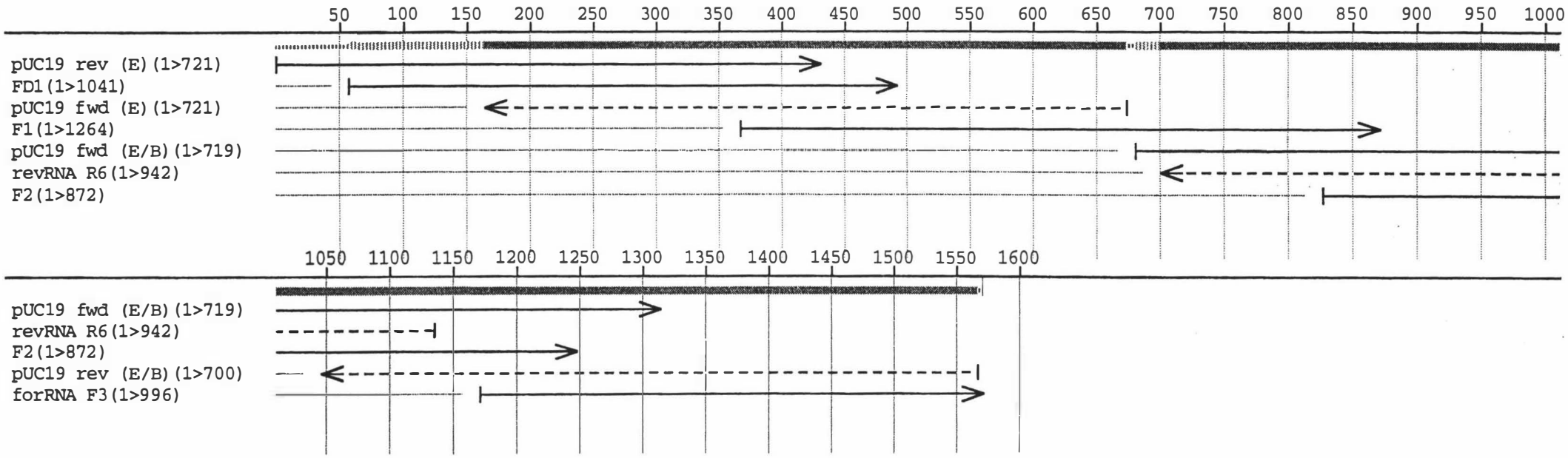
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revRNA R6(1>942)
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S. bovis C14b#1, 16S rRNA

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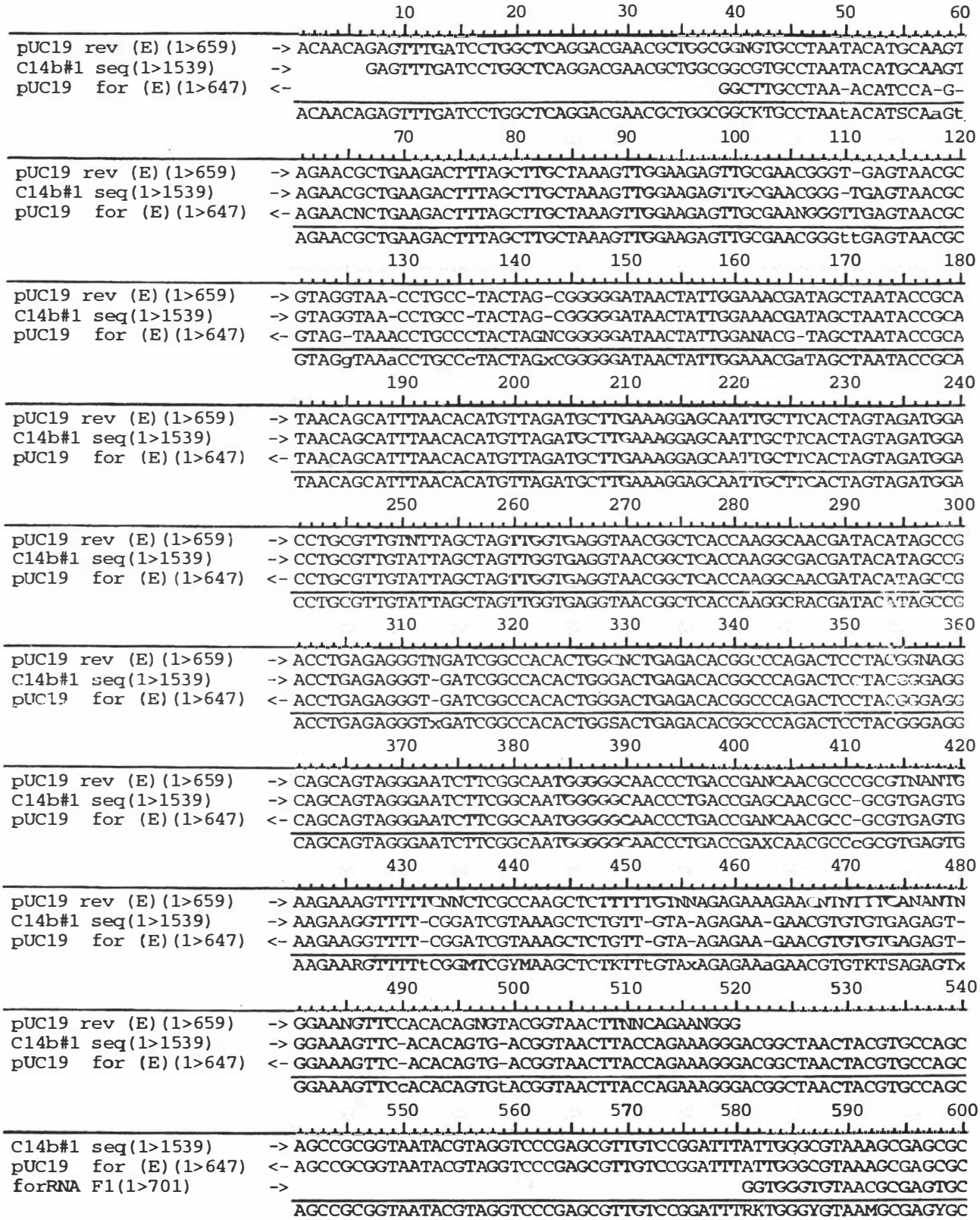
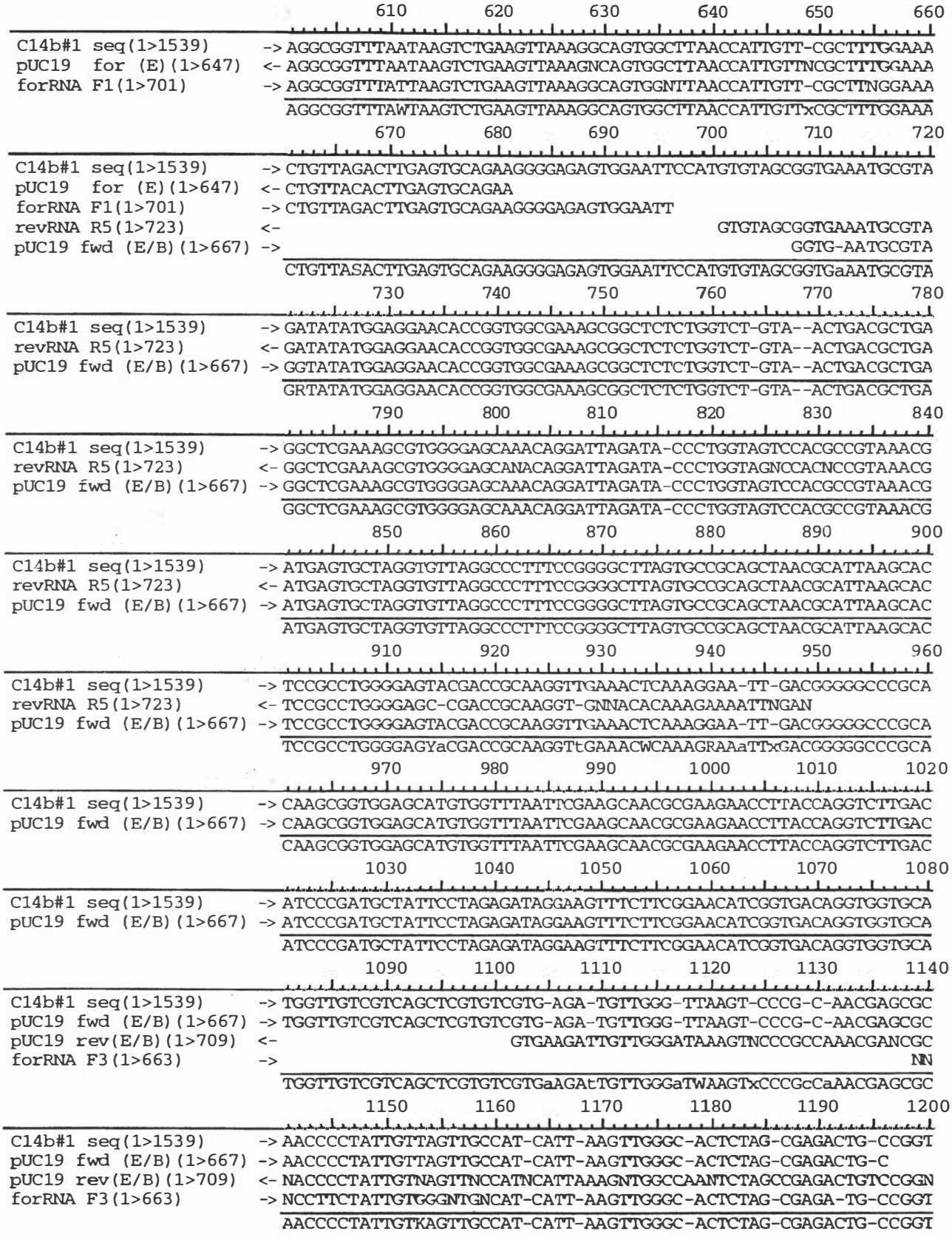
S. bovis C14b#1 sequence alignments

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pUC19 for (E)(1>647) <- AGGCGGTTTAATAAGTCTGAAGTTAAAGNCAGTGGCTTAACCATTGTTNCGCTTTGGAAA
forRNA F1(1>701) -> AGGCGGTTTATTAAGTCTGAAGTTAAAGGCAGTGGNTTAACCATTGTT-CGCTTNGGAAA
AGGCGGTTTAWTAAGTCTGAAGTTAAAGGCAGTGGCTTAACCATTGTTxCGCTTTGGAAA
670 680 690 700 710 720
C14b#1 seq(1>1539) -> CTGTTAGACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTA
pUC19 for (E)(1>647) <- CTGTTACACTTGAGTGCAGAA
forRNA F1(1>701) -> CTGTTAGACTTGAGTGCAGAAGGGGAGAGTGGAATT
revRNA R5(1>723) <- GTGTAGCGGTGAAATGCGTA
pUC19 fwd (E/B)(1>667) -> GGTG-AATGCGTA
CTGTTASACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGaAATGCGTA
730 740 750 760 770 780
C14b#1 seq(1>1539) -> GATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCT-GTA--ACTGACGCTGA
revRNA R5(1>723) <- GATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCT-GTA--ACTGACGCTGA
pUC19 fwd (E/B)(1>667) -> GGTATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCT-GTA--ACTGACGCTGA
GRTATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCT-GTA--ACTGACGCTGA
790 800 810 820 830 840
C14b#1 seq(1>1539) -> GGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATA-CCCTGGTAGTCCACGCCGTAAACG
revRNA R5(1>723) <- GGCTCGAAAGCGTGGGGAGCANACAGGATTAGATA-CCCTGGTAGNCCACNCCGTAAACG
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C14b#1 seq(1>1539) -> ATGAGTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAGTGCCGCAGCTAACGCATTAAGCAC
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C14b#1 seq(1>1539) -> TCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA-TT-GACGGGGGCCCGCA
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pUC19 fwd (E/B)(1>667) -> TCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA-TT-GACGGGGGCCCGCA
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ATCCCGATGCTATTCCTAGAGATAGGAAGTTTCTTCGGAACATCGGTGACAGGTGGTGCA
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pUC19 fwd (E/B)(1>667) -> TGGTTGTCGTCAGCTCGTGTCGTG-AGA-TGTTGGG-TTAAGT-CCCG-C-AACGAGCGC
pUC19 rev(E/B)(1>709) <- GTGAAGATTGTTGGGATAAAGTNCCCGCCAAACGANCGC
forRNA F3(1>663) -> NN
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pUC19 fwd (E/B)(1>667) -> AACCCCTATTGTTAGTTGCCAT-CATT-AAGTTGGGC-ACTCTAG-CGAGACTG-C
pUC19 rev(E/B)(1>709) <- NACCCCTATTGTNAGTTNCCATNCATTAAAGNTGGCCAANTCTAGCCGAGACTGTCCGGN
forRNA F3(1>663) -> NCCTTCTATTGTGGGNTGNCAT-CATT-AAGTTGGGC-ACTCTAG-CGAGA-TG-CCGGT
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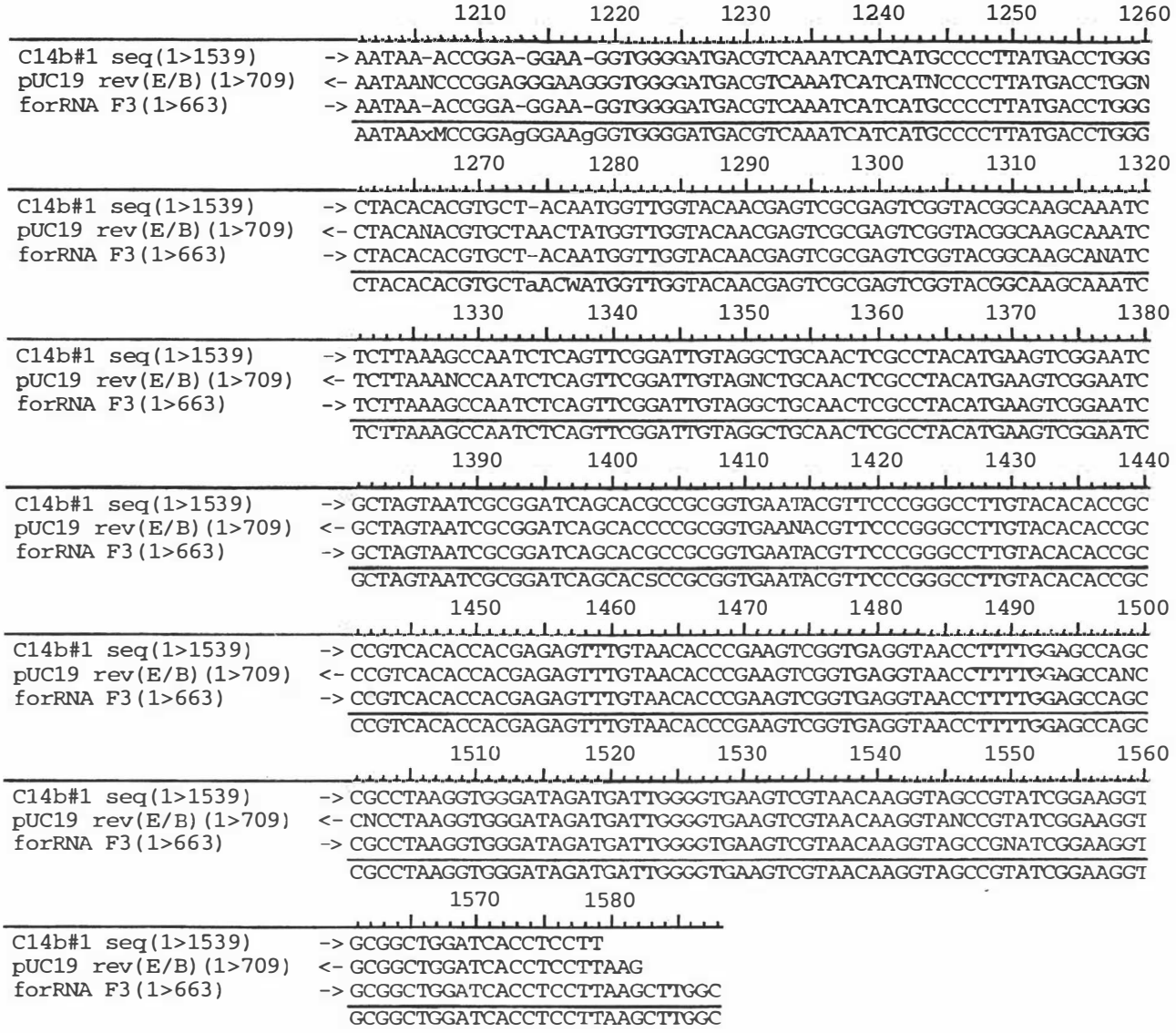
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pUC19 rev(E/B)(1>709) <- AATAANCCCGGAGGGAAGGGTGGGGATGACGTCAAATCATCATNCCCCTTATGACCTGGN
forRNA F3(1>663) -> AATAA-ACCGGA-GGAA-GGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG
AATAAxMCCGGAgGGAAgGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG
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1330 1340 1350 1360 1370 1380
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C14b#1 seq(1>1539) -> GCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
pUC19 rev(E/B)(1>709) <- GCTAGTAATCGCGGATCAGCACCCCGCGGTGAANACGTTCCCGGGCCTTGTACACACCGC
forRNA F3(1>663) -> GCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
GCTAGTAATCGCGGATCAGCACSCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
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forRNA F3(1>663) -> CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGC
CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGC
1510 1520 1530 1540 1550 1560
C14b#1 seq(1>1539) -> CGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGT
pUC19 rev(E/B)(1>709) <- CNCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAGGTANCCGTATCGGAAGGT
forRNA F3(1>663) -> CGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGNATCGGAAGGT
CGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGT
1570 1580
C14b#1 seq(1>1539) -> GCGGCTGGATCACCTCCTT
pUC19 rev(E/B)(1>709) <- GCGGCTGGATCACCTCCTTAAG
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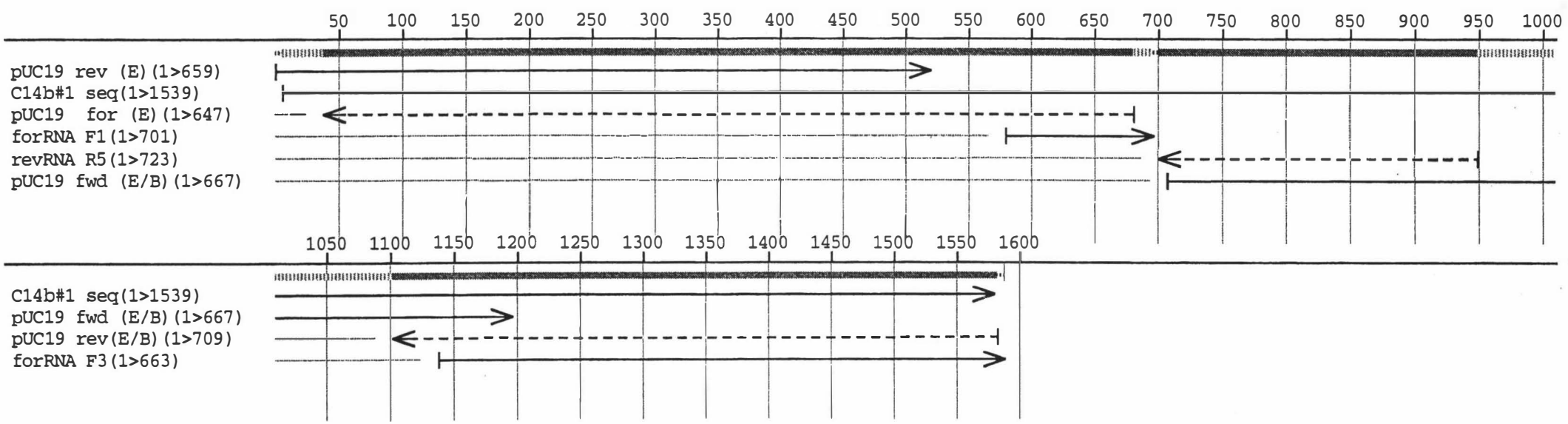
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forRNA F1(1>701)
revRNA R5(1>723)
pUC19 fwd (E/B)(1>667)
1050 1100 1150 1200 1250 1300 1350 1400 1450 1500 1550 1600
C14b#1 seq(1>1539)
pUC19 fwd (E/B)(1>667)
pUC19 rev(E/B)(1>709)
forRNA F3(1>663)

APPENDIX D

Detection of *Clostridium proteoclasticum* in the Rumen using competitive PCR.

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Detection of *Clostridium proteoclasticum* and Closely Related Strains in the Rumen by Competitive PCR

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A competitive PCR technique was used to enumerate the proteolytic bacterium *Clostridium proteoclasticum* from the rumen. A PCR primer, which circumscribes this organism and several closely related strains, was designed for a variable region within their 16S rRNA genes and was used in conjunction with a universal forward primer. This primer pair was tested for specificity against 85 ruminal bacterial strains. An internal control DNA was constructed for use in competitive PCRs and was shown to amplify under the same reaction conditions and with the same amplification efficiency as the target DNA. DNA from a known number of *C. proteoclasticum* cells was coamplified with the internal control to construct a standard curve. Rumen samples were collected from eight dairy cows fed four diets in rotation: high nitrogen, high nitrogen supplemented with carbohydrate, low nitrogen, and low nitrogen supplemented with carbohydrate. DNA extracted from these and spiked with internal control DNA was amplified with the *C. proteoclasticum* primer pair. The relative intensities of the PCR products were used to quantitate the numbers of *C. proteoclasticum* cell equivalents from the rumen samples. The numbers ranged from 2.01×10^6 ml^{-1} to 3.12×10^7 ml^{-1}. There was no significant effect on the numbers of *C. proteoclasticum* detected in rumen samples among cows fed the four diets. The utility of the competitive PCR approach for quantifying ruminal bacterial populations in vivo and the occurrence of *C. proteoclasticum* in forage-fed dairy cows are discussed.

New Zealand ruminants graze a fresh forage diet which is high in protein and low in soluble carbohydrates (10). Up to 50% of the protein available from this diet can be lost due to the rapid microbial breakdown of plant protein (16). Bacteria are thought to be responsible for the majority of this protein degradation in the rumen (8). The proteolytic bacteria in forage-fed New Zealand cattle are dominated by species of the genera *Streptococcus*, *Eubacterium*, and *Butyrivibrio* (2), while a novel, highly proteolytic strain, *Clostridium proteoclasticum*, has also been isolated. To determine the significance of these particular proteolytic bacteria within the New Zealand ruminant, we have set out to develop a sensitive and specific method of quantitation of microbial populations directly from rumen samples.

rRNA probes have been successfully used for the detection and enumeration of bacteria within the rumen (7, 11, 17–20, 27). This technique expresses the abundance of a particular rRNA sequence in relation to total RNA extracted from a sample. However, it is relatively insensitive, only detecting down to 10^6 bacteria per ml of rumen fluid (27). PCR has been used to detect bacteria in many environments (5, 6), and its sensitivity has allowed the detection of a single bacterium (28). Conventional PCR amplifies target DNA exponentially, making it difficult to use the technique in a quantitative manner. Competitive PCR (cPCR), however, has been used to determine bacterial numbers in a range of environments (12, 14, 15). The addition of an internal DNA standard controls for the variation among reactions, allowing reliable PCR quantitation. The internal DNA standard contains the same primer binding sites as the target, and the two DNAs compete for reaction reagents to produce PCR products of different sizes, which can be separated in an agarose gel. The log ratio of intensities of amplified target DNA to internal control DNA is determined by the equation $\log (N_{n1}/N_{n2}) = \log (N_{01}/N_{02}) + n \log (\text{eff}_1/\text{eff}_2)$ (29). If the efficiencies of amplification (eff_1 and eff_2) are equal, the ratio of amplified products (N_{n1}/N_{n2}) is dependent on the log ratio of starting products (N_{01}/N_{02}) (29). Even if the efficiencies of the two reactions are not equal, the values still hold assuming that $\text{eff}_1/\text{eff}_2$ is constant and amplification is in the exponential phase. With this technique, amounts of target DNA can be determined by amplification with known amounts of internal control DNA. The resulting log ratio of intensities of PCR products is compared to standard curves derived from serial dilutions of known target DNA amplified with known amounts of internal control DNA.

C. proteoclasticum is a gram-positive, straight to slightly curved rod which was first isolated from a pasture-grazed cow in New Zealand (4). Its most distinguishing feature is its extremely high proteinase activity, and because of this feature we were interested in quantifying this organism to estimate its contribution to rumen proteolysis. Since its description as a new species, it has become apparent that the 16S rRNA gene (rDNA) sequence of *C. proteoclasticum* is very similar to those of *Butyrivibrio fibrisolvens* NCDO 2435, 2434, 2222, and 2398. However, the description of *C. proteoclasticum* as a new species is justified since *C. proteoclasticum* and these *Butyrivibrio fibrisolvens* strains are phylogenetically closely related to each another (96.9 to 99.5% 16S rRNA sequence similarity) but are distantly related to the *Butyrivibrio fibrisolvens* type strain NCDO 2221^T (93.1 to 94.1% similarity). We have developed a cPCR technique which detects *C. proteoclasticum* and these closely related *B. fibrisolvens* strains directly from rumen samples. PCR primers were designed against a common region of the *C. proteoclasticum* and *B. fibrisolvens* 16S rRNA genes. These primers have been tested for specificity against DNA from 85 rumen bacterial strains, and the sensitivity of detection in a mixed rumen bacterial background has been investigated. We have used this cPCR approach to enumerate *C. proteoclasticum* cell equivalents in rumen samples from dairy cows fed

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TABLE 1. Bacterial strains used in this study

Species	Strain	Source or reference	Reaction with:	
			Universal primers[a]	Specific primers[b]
Butyrivibrio fibrisolvens	C130a, C211, C219	2	+	−
Butyrivibrio fibrisolvens	CF3, H17c	M. P. Bryant, University of Illinois	+	−
Butyrivibrio fibrisolvens	WV1	Laboratory strain	+	−
Butyrivibrio fibrisolvens	OR509	R. Teather, Centre for Food and Animal Research, Agriculture and Agri-food, Canada	+	−
Butyrivibrio fibrisolvens like	C122a, C21b	2	+	−
Clostridium aminophilum	F	J. B. Russell, Cornell University	+	−
Clostridium clostridioforme	ATCC 25537, ATCC 29084	ATCC[c]	+	−
Clostridium proteoclasticum	ATCC 51982	2	+	+
Clostridium sticklandii	SR	J. B. Russell, Cornell University	+	−
Enterococcus faecalis	NCTC 775	S. Flint, New Zealand Dairy Research Institute	+	−
Enterococcus faecalis	68a	Laboratory strain	+	−
Eubacterium cellulosolvens	5494	M. P. Bryant	+	−
Eubacterium limosum	ATCC 8486	M. P. Bryant	+	−
Eubacterium ruminantium		M. P. Bryant	+	−
Eubacterium sp.	C11, C12b, C13b, C14b#2, C118b, C119b, C120a, C124b, C125b, C260	2	+	−
Fibrobacter succinogenes	AC3	M. P. Bryant	+	−
Lachnospira multiparus	ATCC 19307	M. P. Bryant	+	−
Megasphaera elsdenii	T81	M. P. Bryant	+	−
Peptostreptococcus anaerobius	C	J. B. Russell	+	−
Peptostreptococcus productus	SF-50	M. P. Bryant	+	−
Prevotella ruminicola	C21a	2	+	−
Prevotella ruminicola subsp. *brevis*	118b, B_14	M. P. Bryant	+	−
Prevotella ruminicola subsp. *ruminicola*	23	M. P. Bryant	+	−
Ruminococcus albus	7, 8	M. P. Bryant	+	−
Ruminococcus flavefaciens	ATCC 19208, FD1	M. P. Bryant	+	−
Selenomonas ruminantium subsp. *lactilytica*	GA31	M. P. Bryant	+	−
Selenomonas ruminantium subsp. *ruminantium*	ATCC 12561, ATCC 27209	M. P. Bryant	+	−
Streptococcus bovis	JB1	M. P. Bryant	+	−
Streptococcus bovis	NCFB 2476	A. G. Williams, Hannah Research Institute	+	−
Streptococcus bovis	3-31, 3-36, 3-39, 5-21, 7-2, 7-25, 7-26	J. B. Russell	+	−
Streptococcus bovis	A12, A14, A120, A166, A191b, B11, B32a, B314, B315, B327, B337, B342, B346, B348, B350, B360, B372, B382, B385, B395, B396, B398, C14b#1, C17, C123b, C271	2	+	−
Streptococcus bovis	RF-1, RF-2, UDY-KF	Laboratory strains	+	−
Succinomonas amylolytica	ATCC 19206	M. P. Bryant	+	−
Succinovibrio dextrinosolvens	ATCC 27209	M. P. Bryant	+	−

[a] PCR amplification with universal forward and universal reverse primers.
[b] PCR amplification with universal forward primer and S-S-Cprot-0832-a-A-21.
[c] ATCC, American Type Culture Collection, Rockville, Md.

four different diets: high nitrogen, high nitrogen supplemented with carbohydrate, low nitrogen, and low nitrogen supplemented with carbohydrate.

MATERIALS AND METHODS

Bacterial strains and growth. The bacterial strains used are listed in Table 1. Bacteria were grown on CC medium (13), except that the rumen fluid was not preincubated to remove soluble carbohydrates and carbon sources were replaced by either 1.0% (wt/vol) glucose or cellobiose. *Clostridium aminophilum*, *C. sticklandii*, and *Peptostreptococcus anaerobius* were grown in CC medium in which no carbon source was added and tryptone (1.5% [wt/vol]; Difco, Detroit, Mich.) replaced trypticase.

Rumen samples. Eight fistulated, lactating, Friesian dairy cows were fed four different diets in rotation: high nitrogen, high nitrogen with carbohydrate, low nitrogen, and low nitrogen with carbohydrate. Ryegrass pasture, containing less than 5% clover, was top dressed with 20 kg of nitrogen (as urea) per ha for low-nitrogen diets and 90 kg of nitrogen per ha for high-nitrogen diets. Urea was applied 21 to 28 days before cutting, and the nitrogen was 2.11 and 2.82% of dry matter for the low- and high-nitrogen diets, respectively. Carbohydrate was

supplied as a 50:50 mixture of dextrose and corn flour on an energy basis and was drenched at 9:00 a.m., 11:00 a.m., 4:00 p.m., and 6:00 p.m., supplying 10% of the minimum energy intake. The cows were fed at 9:00 a.m. and 4:00 p.m. and were maintained on each diet for 12 days before the samples were taken. Whole rumen samples of approximately 250 ml were collected before the last 4:00 p.m. feed, frozen immediately in liquid nitrogen, and stored at −80°C until DNA extraction. Immediately before DNA extraction, the samples were thawed in a 37°C water bath and diluted with an equal weight of mineral salts (MS) buffer (3) before homogenization in a Sorvall tissue homogenizer (Du Pont Co., Wilmington, Del.).

DNA extraction. General techniques of DNA precipitation and phenol-chloroform extractions were performed as described by Sambrook et al. (26). For determination of primer specificity, bacterial cultures were grown overnight and DNA was extracted by the enzymatic lysis procedure described by Saito and Miura (25). To eliminate possible bias introduced by enzymatic cell wall lysis and to maximize DNA extraction efficiencies, physical disruption was used to extract DNA from rumen samples and from *C. proteoclasticum* for sensitivity experiments and standard curve construction. Unless otherwise stated, DNA was extracted from triplicate samples. A 1-ml volume of homogenized rumen contents or 10^{10} *C. proteoclasticum* cells was added to 1.2 g of sterile zirconia-silica beads (Biospec Products) followed by centrifugation at 12,000 × *g* for 10 min at room temperature. The pellet and beads were rinsed twice in saline-EDTA solution (0.15 M NaCl, 0.1 M EDTA) before final resuspension in 750 μl of saline-EDTA. Physical disruption was then performed with a Mini-beadbeater (Biospec Products) at maximum speed for two intervals of 2 min each, with a 1-min incubation on ice between each treatment. Phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed, and the mixture was centrifuged at 12,000 × *g* for 5 min at room temperature. The aqueous phase was removed, and the interface was re-extracted with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The combined aqueous phases were repeatedly extracted with the phenol-chloroform-isoamyl alcohol until no protein remained at the interface. A final chloroform-isoamyl alcohol extraction was performed before the nucleic acids were precipitated with ethanol and centrifuged at 10,000 × *g* for 20 min at 4°C. The air-dried pellet was resuspended in TE buffer, RNase A (1 mg ml^{-1} [final concentration]) was added, and the mixture was incubated at 37°C for 60 min. RNase A was removed by phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation and centrifugation. The air-dried DNA pellet was resuspended in TE buffer and was stored at −20°C until required. DNAzol reagent (Gibco BRL, Life Technologies, Auckland, New Zealand) was used for chemical extraction of DNA. Cells were suspended in 1 ml of DNAzol reagent before being subjected to bead beater treatment as described above. The DNA was then precipitated as specified by the manufacturer. The concentration and purity of the DNA were determined spectrophotometrically by measuring the absorbances at 260 and 280 nm ($A_{260/280}$) with a Spectramax microplate spectrophotometer (Molecular Dynamics).

To check that there was no amplification of plant material with the primer pair, DNA was extracted from 20 g of pasture plant tissue. This plant tissue was diluted with 4 volumes of MS buffer to ensure complete homogenization. The DNA was extracted by the mechanical disruption method described above.

PCR primers and amplification. The primers used for the amplification of the 16S rRNA genes were as follows: universal forward primer (S-*-Univ-0008-a-S-19; 5′ GAG TTT GAT CCT GGC TCA G 3′), universal reverse primer (S-*-Univ-1528-a-A-17; 5′ AAG GAG GTG ATC CAG CC 3′), and the *C. proteoclasticum* primer (S-S-Cprot-0832-a-A-21; 5′ CTG AAT GCC TAT GGC ACC CAA 3′). The S-S-Cprot-0832-a-A-21 primer was screened for specificity with the PROBE CHECK program of the Ribosomal Database Project (21) and the BLAST (Basic Local Alignment Search Tool) facility at the National Center for Biotechnology. PCR amplification of *C. proteoclasticum* DNA produces an 830-bp product when amplified with the universal forward primer and S-S-Cprot-0832-a-A-21. Because the S-S-Cprot-0832-a-A-21 primer also detects some closely related *B. fibrisolvens* strains in rumen samples, in these instances cell numbers are expressed as *C. proteoclasticum* cell equivalents.

The PCR mixtures contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 1 μM each primer, and 0.5 U of *Taq* DNA polymerase (Gibco BRL). The PCRs were performed in a final volume of 20 μl sealed in a capillary tip, and thermocycling was carried out in a model FTS-1 capillary thermal sequencer (Corbett Research, Sydney, Australia). The PCR amplification conditions for S-S-Cprot-0832-a-A-21 and the universal forward primers were as follows: denaturation at 95°C for 3 min followed by 6 cycles of 95°C for 30 s, 62°C for 15 s, and 72°C for 30 s and 25 cycles of 95°C for 15 s, 62°C for 5 s, and 72°C for 30 s, with a final cycle of 72°C for 3 min. Amplification with the universal forward and reverse primers differed only in the annealing temperature, which was 55°C. PCR products were separated by electrophoresis in agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

Construction of the internal control. The 830-bp PCR product from *C. proteoclasticum* DNA amplified with the universal forward and S-S-Cprot-0832-a-A-21 primers contains two *Alu*I sites (Fig. 1). To produce an *Alu*I deletion, the 830-bp fragment was digested with *Alu*I as specified by the manufacturer (Boehringer, Mannheim, Germany) and the restriction endonuclease was removed with phenol treatment followed by ethanol precipitation. The *Alu*I fragments were ligated with T4 DNA ligase (Gibco BRL), 2 μl of the ligation reaction was used in a PCR with the universal forward and S-S-Cprot-0832-a-A-21 primers, and the products were separated by agarose gel electrophoresis. One of the PCR products was a 480-bp fragment expected from the deletion of the 350-bp internal *Alu*I fragment. The 480-bp fragment was purified from the gel and used as an internal control for the cPCRs. The concentration of the internal control was estimated from photographs of the gel to be approximately 100 ng ml^{-1}. However, the DNA concentration was too low to obtain an accurate $A_{260/280}$ reading, and so the internal control was expressed as a dilution of the concentrated mixture.

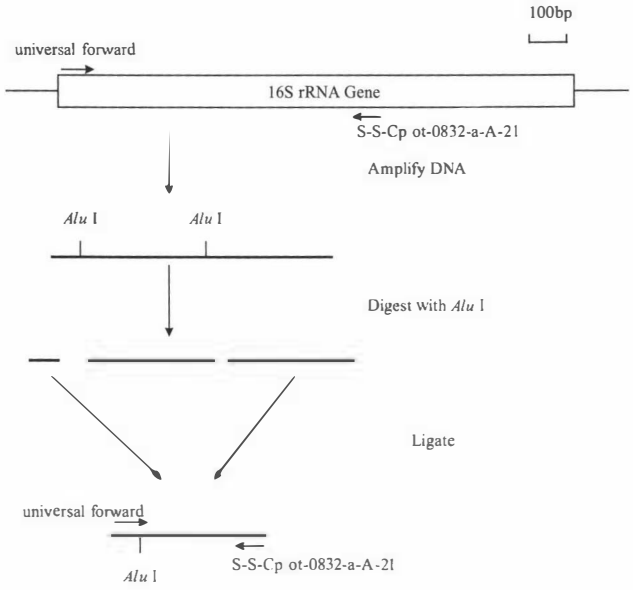

FIG. 1. Construction of the internal control. The 830-bp PCR product was digested with *Alu*I to give three fragments of 90, 350, and 390 bp. The fragments were ligated and PCR amplified. The 480-bp product was purified and used as the internal control.

Preparation of *C. proteoclasticum* cells for sensitivity testing. *C. proteoclasticum* was grown in 100-ml overnight cultures, and a sample was counted by phase-contrast microscopy with a WSI counting chamber (Weber Scientific International Ltd., Middlesex, England). The cells were pelleted by centrifugation and resuspended to a final concentration of 10^{10} cells ml^{-1}. DNA extractions were performed on 10^{10} cells; 10-fold serial dilutions of this DNA were amplified to determine the detection limit.

Quantitation of PCR products. PCR products were quantitated by photographing agarose gels with Polaroid 665 film (Polaroid, St. Albans, England), which produces a negative image of the photograph. The negative was scanned with a GS-670 imaging densitometer (Bio-Rad, Hercules, Calif.) and analyzed with Molecular Analyst software version 1.4 (Bio-Rad). To correct for differences in the fluorescence of ethidium bromide-stained PCR fragments of different sizes (23), the intensity of the internal control was multiplied by the ratio 830/480.

RESULTS

Primer specificity and sensitivity. Alignment of the *C. proteoclasticum* 16S rDNA sequence with other sequences from the Ribosomal Database Project and closely related *B. fibrisolvens* sequences retrieved from BLAST searches identified a region at bp 832 to 851 (*E. coli* numbering) that was common to *C. proteoclasticum* and *B. fibrisolvens* NCDO 2435, 2222, 2398, and 2434. A primer, S-S-Cprot-0832-a-A-21, complementary to the sense strand of this region facing the 5′ end of the 16S rRNA gene was designed. This enabled it to be used with the universal forward primer in PCRs to generate an 830-bp product. The primer pair was tested for specificity against DNA from 85 bacterial strains, mostly of rumen origin (Table 1). Only *C. proteoclasticum* genomic DNA amplified with these two primers at an annealing temperature of 62°C (Table 1); closely related strains (*B. fibrisolvens*-like C21b and C122b) failed to amplify under these conditions. At an anneal-

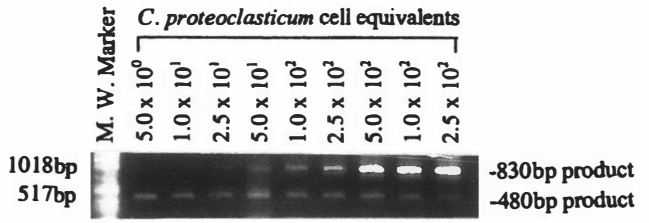

FIG. 2. Detection limit of cPCR. DNA extracted from 10^{10} *C. proteoclasticum* cells ml^{-1} was serially diluted and coamplified with a 2×10^6 dilution of the internal control to determine the detection limit of the cPCR assay. The results are expressed as *C. proteoclasticum* cell equivalents based on the amount of DNA extracted per cell.

ing temperature of 60°C, some amplification occurred from unrelated *Enterococcus faecalis* NCTC 775 and *Streptococcus bovis* RF-2. This nonspecific amplification was eliminated once the annealing temperature was raised to 62°C. The DNA from each of the rumen strains was also tested with the universal forward and reverse primers at an annealing temperature of 55°C. All the strains produced a PCR fragment of approximately 1,500 bp, corresponding to the approximate size of the 16S rRNA gene, demonstrating that the DNA from each strain was amplifiable.

The sensitivity of the S-S-Cprot-0832-a-A-21 primer in PCRs was investigated by amplification of a serial dilution of DNA from known numbers of *C. proteoclasticum* cells. The results show that 50 fg of DNA (the equivalent of DNA from 25 *C. proteoclasticum* cells) was the lower limit of detection when coamplified with a 2×10^6 dilution of the internal control DNA (Fig. 2).

DNA extraction. To determine the most efficient method of recovering DNA from bacterial samples, 10^{10} *C. proteoclasticum* cells were subjected to three methods of DNA extraction: enzymatic cell lysis, chemical extraction, and physical disruption. $A_{260/280}$ readings demonstrated that physical disruption

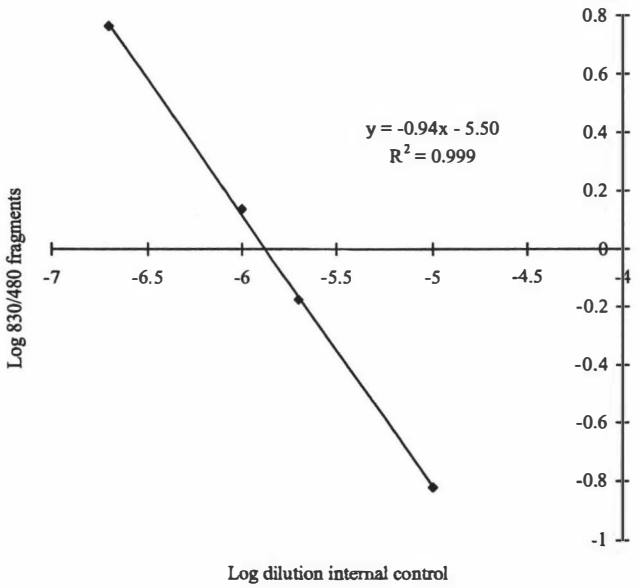

FIG. 3. Internal-control amplification efficiency. Dilutions of the internal control were coamplified with DNA from 10^3 *C. proteoclasticum* cells, and the ratio of the intensities of internal control to the target DNA was plotted against the dilution of the internal control on a log scale.

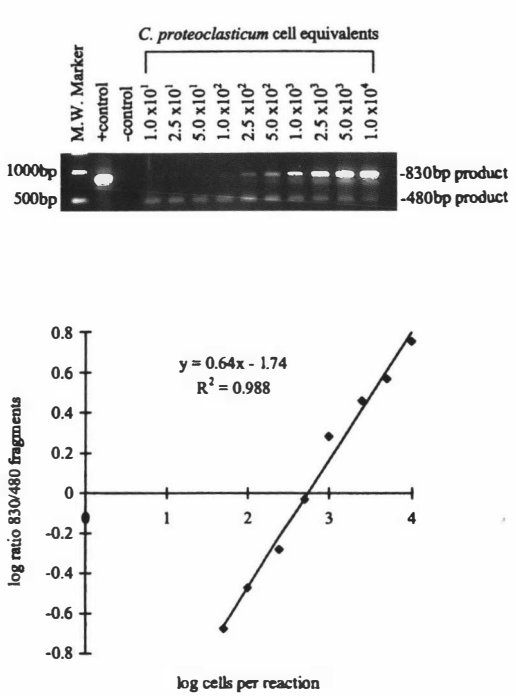

FIG. 4. Standard curve construction. (a) DNA extracted from 10^{10} *C. proteoclasticum* cells ml^{-1} was serially diluted and coamplified with a 10^6 dilution of the internal control. The results are expressed as *C. proteoclasticum* cell equivalents based on the amount of DNA extracted per cell. (b) Ratios of the intensities of internal control to target DNA were quantitated by scanning densitometry of negative images of Polaroid photographs of ethidium bromide-stained gels.

was the most efficient method of extraction, recovering 67 μg of DNA from 10^{10} cells. Enzymatic lysis and chemical extraction recovered 43 and 1.01 μg of DNA per 10^{10} cells, respectively.

Internal-control amplification efficiency. The relative amplification efficiencies of target and internal-control DNAs can be determined from a plot of the ratio of log target intensity to internal control intensity against the log concentration of internal control DNA. Coamplification of DNA from 10^3 *C. proteoclasticum* cells with dilutions of the internal control (Fig. 3) results in a line with a slope of 0.94 and a regression of 0.999. This indicates equivalent amplification efficiencies of the target and control DNAs. The line intersects the x axis at -5.9, indicating that the optimal dilution of internal control for detection of 10^3 *C. proteoclasticum* cells is 1.26×10^6.

Standard curve. Since cPCR is most accurate when the target and internal control are coamplified in equimolar proportions, it was necessary to determine the optimal internal-control concentrations to use with DNA extracted from rumen samples. Dilutions of internal control were coamplified with DNA from selected samples, and the optimal dilution of the internal control was found to be 10^{-6}. This concentration of internal control was used to construct a standard curve by coamplification with *C. proteoclasticum* DNA extracted from a known number of cells (Fig. 4). The results show that DNA from 1×10^4 to 5×10^1 cells gave a linear response and could be used for quantitation of samples within this range.

Detection of *C. proteoclasticum* added to rumen fluid. To test whether *C. proteoclasticum* could be detected by cPCR within a background of nonspecific DNA from rumen fluid, samples of rumen contents were spiked with known numbers of *C. pro-*

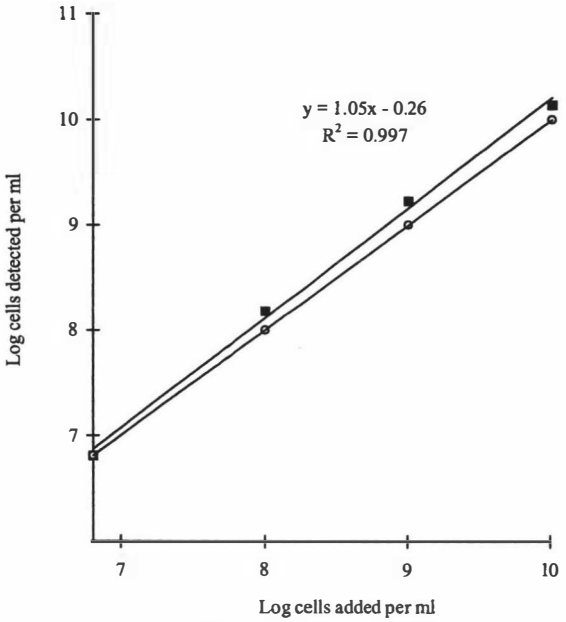

FIG. 5. In vivo detection of *C. proteoclasticum*. Rumen samples were spiked with known numbers of *C. proteoclasticum* cells. DNA was extracted from these mixtures and assayed for the presence of *C. proteoclasticum*. Solid squares denote numbers of *C. proteoclasticum* cell equivalents detected in each sample. Open circles denote $y = x$. The y intercept of the line denotes no *C. proteoclasticum* cells added. This represents a background population of *C. proteoclasticum* and closely related *B. fibrisolvens* strains of 6.46×10^6 ml^{-1}.

teoclasticum cells. DNA from each of the samples was coamplified under optimal cPCR conditions, and the results show a linear relationship between the number of cells added and the number of cells detected. The assay slightly overestimated the number of *C. proteoclasticum* cells added compared to the ideal ($y = x$), and this was particularly evident at the higher concentrations of cells (Fig. 5). A population of 6.25×10^6 *C. proteoclasticum* cell equivalents ml^{-1} was detected in the unspiked rumen samples.

Detection of *C. proteoclasticum* and closely related strains in vivo. To examine the application of the cPCR approach to determining bacterial numbers directly from animals, rumen samples were collected from eight lactating dairy cows fed four different diets in rotation. The number of *C. proteoclasticum* cell equivalents detected ranged from 2.01×10^6 to 3.12×10^7 ml^{-1} of rumen contents (Fig. 6). Within individual animals there were significant responses to diet, but overall there was no significant difference between the diets.

DISCUSSION

C. proteoclasticum is a proteolytic bacterium that was isolated from the rumen contents of a forage-fed cow (2). It has a predominately serine-type proteinase activity (3) but also some cysteine- and metallo-type proteinase activity. It seems most likely to be involved with primary hydrolysis of feed protein, but the significance of *C. proteoclasticum* to rumen microbial ecology has not yet been determined. Since no suitable selective media are available for enumeration of *C. proteoclasticum*, we set out to develop a method to quantify this organism directly from rumen samples.

PCR is an extremely sensitive and specific method for the amplification of DNA, and when it is used in conjunction with an internal DNA control, the products of the amplification reactions can be quantified. This technique, cPCR, was originally developed for quantitation of human immunodeficiency virus type 1 3B long terminal repeat DNA (29). Its use has since been extended to bacterial quantitation in the environment (12, 14, 15). In the present study, a cPCR method was developed to quantify *C. proteoclasticum* and closely related strains from rumen samples. PCR primers, based on both conserved and hypervariable regions of the 16S rRNA gene, were used to amplify DNA in the presence of an internal control which allowed quantitation of the PCR products. The primer pair used circumscribes *C. proteoclasticum* and four closely related *B. fibrisolvens* strains and did not amplify DNA from any other bacterium tested. The specificity of the assay stems from the selection of 16S rDNA as the target for amplification and the primer design. The semiconserved nature of rRNA genes allows the use of regions of hypervariable sequence as targets for group-, genus-, species-, and strain-specific amplification (22). The primer was designed to match, as closely as possible, the length, G+C content, and T_m of the universal forward primer so that the forward primer "anchored" the PCR at the 5′ end of the 16S rRNA gene. This approach limits the range of suitable specific primers which can be designed within a particular region but avoids the need to design two specific primers for a given organism. Using the 16S rRNA gene as the amplification target also allows primers to be checked against the existing entries in DNA sequence databases, thereby reducing the effort needed to test primer specificity.

A critical factor in ensuring the accuracy of cPCR is to demonstrate that coamplifications of the target and internal control are equivalent (24). This can be done by plotting the log ratio of target to internal-control DNA intensities against the log dilution of internal-control DNA. Coamplification of *C. proteoclasticum* DNA with dilutions of internal control gave a straight line with a slope of −0.94, indicating that the amplification efficiencies of the two DNAs are essentially equal. The small deviation from equivalence may be due to a slightly more efficient amplification of the smaller internal control (480 bp) than of the target (830 bp). This effect was taken into account by using standard curves to relate the log ratio of target to internal control to the log of cell numbers. These standard curves also account for the rRNA gene copy number, which can vary anywhere from 2 to 10 copies per genome (1, 9). Each standard curve, generated by coamplification of a single dilution of internal control with DNA from different numbers of *C. proteoclasticum* cells, gives a substantial working range of cell numbers of approximately 10^3- to 10^4-fold. Indeed, in the application of the assay to rumen samples, only a single standard curve was required to encompass the entire range of *C. proteoclasticum* numbers encountered in differently fed dairy cows.

In a similar manner, dilutions of internal control can be used to determine the absolute sensitivity of the assay. Theoretically, the detection of one copy of the target sequence is possible (28). However, in cPCR, the target and internal control compete for amplification reagents, reducing the sensitivity of detection. In our assay, DNA from the equivalent of 25 *C. proteoclasticum* cells could be detected when coamplified with a 2×10^6 dilution of the internal control. However, it was found that a 100-fold dilution was necessary to overcome an inhibitory effect in DNA extracted from rumen samples. Thus, in practice, the sensitivity of the assay is limited to 2.5×10^3 cells. The exact nature of the inhibitory factor is not known, but high $A_{260/280}$ readings from these DNA samples indicate that it is not proteinaceous and that it may be a water-soluble polysaccharide or polyphenolic compound similar in nature to the humic acids described by Leser (14) as interfering with the

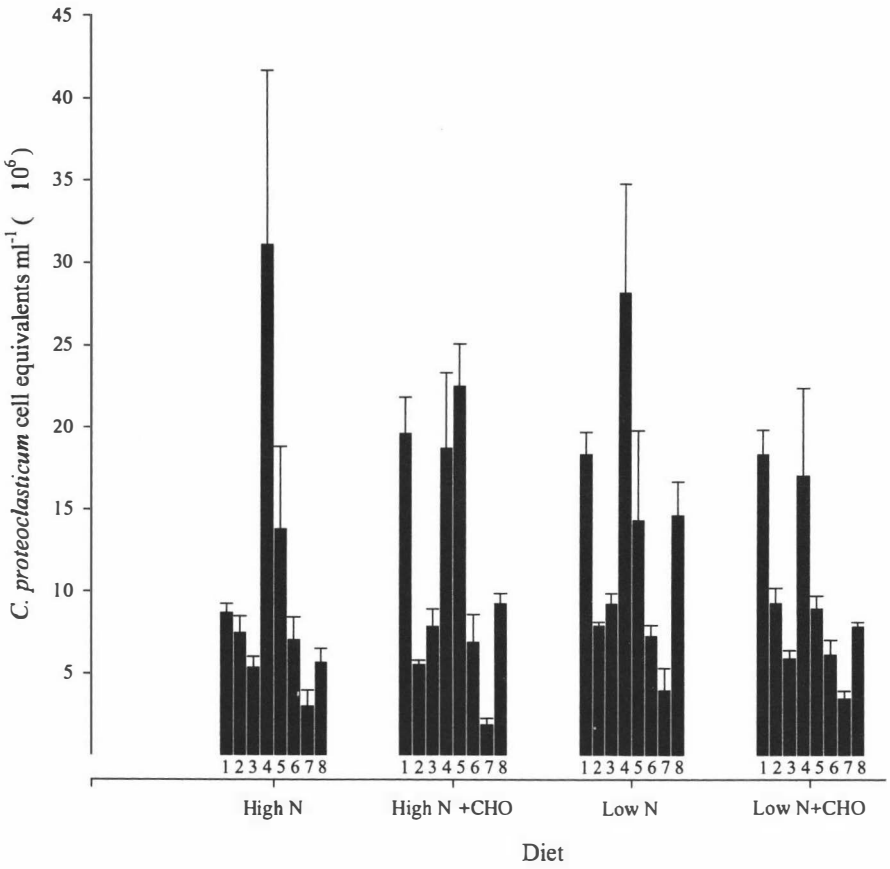

FIG. 6. Populations of *C. proteoclasticum* and closely related *B. fibrisolvens* strains in dairy cows under four different feeding regimens. Numbers 1 through 8 represent cows numbered 709, 710, 727, 788, 1758, 8702, 9754, and 9775, respectively. The results are the means of triplicate determinations and error bars represent standard error of the mean. The diets were fed in rotation, and rumen samples were taken during each feeding regime. CHO, carbohydrate.

PCRs. The level of detection compares favorably with that in similar studies of bacterial populations from other environments. Leser (14) detected DNA from 40 *Pseudomonas* cells in cPCR assays of samples from a marine environment. Radiolabelled oligonucleotides used to probe rRNA from rumen bacteria (7, 11, 17–20, 27) are less sensitive, being able to detect 0.01% of the total rumen population, or approximately 10^6 cells ml^{-1} (27). It should be noted that the cPCR technique does not discriminate between living and dead cells and therefore is likely to overestimate viable populations. This is in contrast with oligonucleotide probing, where microbial abundance is expressed in terms of a proportion of total rRNA. Since the rRNA content in cells changes according to the growth phase, this technique provides an approximation of relative cell numbers (20), which reflects their contribution to total metabolic activity (27). However, the estimation of total rRNA abundance depends on universal probes, and recent evidence suggests that domain-specific variations in dissociation temperatures of universal probes could lead to significant biases in quantifying microbial populations from environmental samples (30). Estimates of both absolute cell numbers and the percent contribution to total rRNA may be the best approach to gain an accurate assessment of the importance of microbial populations in environmental samples.

After the development of the cPCR assay in vitro, it was necessary to test its ability to detect *C. proteoclasticum* cells in the complex mixture of plant and microbial DNA present in rumen fluid. Mechanical disruption was chosen for DNA extraction from rumen contents since the efficiency of extraction was greatest with bead beating followed by phenol-chloroform extractions. Also, this method allowed entire rumen contents to be sampled, thereby enabling the quantitation of populations adherent to plant tissue. Previous methods have sampled only filtered rumen contents (7, 17, 27). The addition of *C. proteoclasticum* cells to rumen fluid followed by cPCR quantitation demonstrated the usefulness of the technique in vivo. There was good correlation between the number of cells added and those detected by the assay. A slight overestimation of absolute numbers of cells compared to the ideal ($y = x$) is probably due to the resident population of *C. proteoclasticum* and closely related *B. fibrisolvens* strains in the rumen fluid used.

When the assay was applied to rumen samples collected from animals fed diets differing in nitrogen and carbohydrate content, the *C. proteoclasticum* cell equivalents detected ranged from 2.01×10^6 cells per ml in the carbohydrate-supplemented, high-nitrogen diet to 3.12×10^7 cells per ml in the high-nitrogen diet. These numbers represent the population of *C. proteoclasticum* and closely related *B. fibrisolvens* strains present in the samples and are consistent with the original isolation of *C. proteoclasticum* from a 10^8 dilution of rumen contents (4). These results indicate that this group of organisms is common among forage-fed ruminants in New Zealand. Within individual animals, the diet had some effects on *C. proteoclasticum* cell equivalents, but overall, between the animals there were no significant differences (Fig. 6). The relatively

small range of cell numbers detected indicates that the population is stable and unresponsive to changes in dietary content. This is a little surprising since one might expect the proteolytic population of *C. proteoclasticum* to be influenced by nitrogen supply. However, the population density of *C. proteoclasticum* does not necessarily reflect its overall contribution to proteolytic activity, which may well vary significantly under the conditions tested. Previously, it was shown that *C. proteoclasticum* has a high chymotrypsin-like proteinase activity (3), and, based on its specific activity for the artificial chymotrypsin substrate *N*-succinyl alanine-alanine-proline-phenylalanine-*p*-nitroanilide and the populations detected in this study, it may be responsible for up to 20% of this type of activity in forage-fed dairy cows. More detailed investigations of specific *C. proteoclasticum* proteinase activities are required before the contribution of this organism to ruminal protein breakdown can be estimated more precisely.

We have found cPCR to be an easy, accurate, and reliable method of bacterial quantitation. Collection of rumen samples, DNA extraction, preliminary PCR amplification with internal control dilutions, and cPCR followed by scanning densitometry can be accomplished within 10 h. Testing primers for specificity and constructing internal DNA controls are the most time-consuming steps in developing the method, but once these have been carried out, they need not be repeated. The precision of the technique is good, with the coefficient of variation between replicate PCRs of the same sample averaging 2.5% and that between samples from the same animal averaging 7.5%. The technique combines the specificity of 16S rDNA-targeted oligonucleotides with the sensitivity of PCR in a format which allows quantitation of bacterial populations from a complex microbial ecosystem. The technique is currently being extended to other protein-degrading genera and will eventually allow us to examine how proteolytic bacterial populations are influenced by changes in the rumen ecosystem.

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