

# Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*

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## Abstract

Competitive PCR assays were developed for the enumeration of the rumen cellulolytic bacterial species: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. The assays, targeting species-specific regions of 16S rDNA, were evaluated using DNA from pure culture and rumen digesta spiked with the relevant cellulolytic species. Minimum detection levels for *F. succinogenes*, *R. albus* and *R. flavefaciens* were 1–10 cells in pure culture and  $10^{3-4}$  cells per ml in mixed culture. The assays were reproducible and 11–13% inter- and intra-assay variations were observed. Enumeration of the cellulolytic species in the rumen and alimentary tract of sheep found *F. succinogenes* dominant ( $10^7$  per ml of rumen digesta) compared to the *Ruminococcus* spp. ( $10^{4-6}$  per ml). The population size of the three species did not change after the proportion of dietary alfalfa hay was increased. All three species were detected in the rumen, omasum, caecum, colon and rectum. Numbers of the cellulolytic species at these sites varied within and between animals. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Competitive PCR; Rumen bacteria; Cellulolytic species; 16S rDNA; *Fibrobacter*; *Ruminococcus*

## 1. Introduction

Anaerobic rumen fibrolytic bacteria, protozoa and fungi degrade fibrous material, allowing ruminants to utilize plant fiber for nutrition. Bacteria are the most numerous of these microorganisms and play a major role in the biological degradation of dietary fiber. *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* are presently recognized as the major cellulolytic bacterial species found in the rumen [1]. Probe hybridization methods for enumeration of these cellulolytic species have already been established and applied to analysis of digesta from the rumen [2,3] and equine caecum [4]. However, RNA-targeted probing methods cannot detect target species when numbers are <0.01% of all bacteria present. This technique is also laborious compared with PCR-based methodologies.

Competitive PCR (cPCR) has the ability to enumerate-

targeted bacteria with high sensitivity [5] and has been used to analyze various environmental samples, such as water [6], soil [7] and rumen digesta [8]. This technique is both reliable and simple to perform. A cPCR assay for tracking a genetically modified rumen bacterium commonly used as a host strain for transforming *Butyrivibrio fibrisolvens* [10–12] has been described [9].

cPCR assay development for the cellulolytic rumen bacterial species *F. succinogenes*, *R. albus* and *R. flavefaciens* is described in this communication. Variations in population sizes of these species can be accurately detected using this technique. Increased knowledge concerning the rumen cellulolytic bacterial population will allow insight into the fiber digestion capabilities of ruminant animals.

## 2. Materials and methods

### 2.1. Bacterial strains, growth conditions and DNA isolation

The bacterial strains used are shown in Table 1. *F. succinogenes* GC5 and *Fibrobacter intestinalis* LH1 were purchased from the American Type Culture Collection and

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strains of *B. fibrisolvens* were donated from Drs. R.J. Forster and R.M. Teather of Lethbridge Research Centre, Lethbridge, AB, Canada. All other ruminal strains were donated from Dr. H. Minato, Ibaraki University, Japan. Bacteria were anaerobically cultured at 37°C using a pre-reduced rumen fluid-based RGC medium, containing glucose, cellobiose, maltose and starch (0.2% each) as carbon sources [13]. Isolation of bacterial DNA, from harvested overnight cultures, was carried out as described by Purdy et al. [14]. In brief, 0.15–0.35 ml of culture was shaken with glass beads, SDS and phenol. Extracted DNA was then purified using hydroxyapatite and gel filtration columns. DNA was fluorescently quantified (DyNA Quant, Hoefer) and used for PCR.

## 2.2. Primers and PCR

Species-specific PCR primers (Table 1) used to amplify partial 16S rDNA regions (target DNA) were chosen from the literature [15] (*R. albus*) or newly designed (*F. succinogenes* and *R. flavefaciens*) using Oligo (MBInsights) and Genetyx software (Software Development, Tokyo). Primers for *F. succinogenes*, Fs219f (5'-GGT ATG GGA TGA GCT TGC-3') and Fs654r (5'-GCC TGC CCC TGA ACT ATC-3'), were selected to allow amplification (446-bp product) of all 10 *F. succinogenes* strains deposited in GenBank. *R. albus* primers, Ra1281f (5'-CCC TAA AAG CAG TCT TAG TTC G-3') and Ra1439r (5'-CCT CCT TGC GGT TAG AAC A-3'), were chosen from previously published sequences that demonstrated species-specific amplification (175-bp product) [15]. *R. flavefaciens* primers, Rf154f (5'-TCT GGA AAC GGA TGG TA-3') and Rf425r (5'-CCT TTA AGA CAG GAG TTT ACA A-3'), were also selected to allow species-specific amplification (295 bp) of all seven *R. flavefaciens* strains deposited in GenBank. PCR conditions for *F. succinogenes* were as follows: 30 s at 94°C for denaturing, 30 s at 60°C for annealing and 30 s at 72°C for extension (48 cycles), except for 9 min denaturation in the first cycle and 10 min extension in the last cycle. A gradually activated Taq polymerase (AmpliTaQ Gold, ABI) was used for this hot start-time release PCR. Amplification of 16S rDNA for the other two species was carried out similarly except an annealing temperature of 55°C was used.

## 2.3. Competitor construction

Specific competitor sequences, to be used as internal controls, were constructed for each cPCR assay. Amplified target DNA of a type strain for each species (*F. succinogenes* S85, *R. albus* 7 or *R. flavefaciens* C94) was cloned into a TA plasmid vector (Invitrogen). Restriction and ligation reactions were used to increase or decrease the length of the target DNA in the plasmid to provide competitor DNA for the cPCR reaction. *F. succinogenes* competitor DNA was produced by removing a *SspI/SmaI* frag-

ment to yield a shorter fragment (205 bp). Competitor DNA for *R. flavefaciens* (207 bp) was produced similarly by removing a *HincII/SspI* fragment. Competitor DNA for *R. albus* (284 bp) was produced by inserting 109 bp of *R. albus* DNA into the *SmaI* site of the target DNA. Competitor DNA was gel-purified and quantified prior to use.

## 2.4. cPCR reaction and data processing

Each competitor was serially diluted and co-amplified by PCR with total DNA from each pure culture or digestive sample. The cPCR products generated were separated on a 2.5% Metaphor (FMC BioProducts) agarose gel containing ethidium bromide, and photographed. The negatives (Polaroid 665) were scanned, along with a standard OD tablet (Bio Image), and band intensities were measured using image analysis software (NIH image 1.57) to determine if co-amplification occurred with equal efficiency. The quantity of competitor added and the ratio of amplified target to amplified competitor were plotted using log scales. The target present in the initial sample was estimated by interpolation using a calculated regression equation. The cPCR assay value was converted into a cell equivalent value by dividing the assay value by the target 16S rDNA concentration in a single cell of each species (experimentally determined).

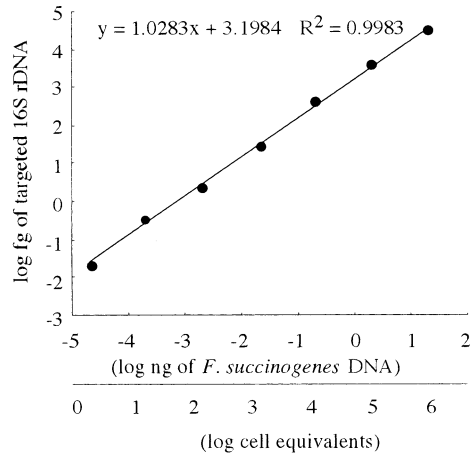
## 2.5. Evaluation of assays and application

Assay sensitivity was assessed by determination of the minimum detection levels of each bacterial target DNA. Assay reproducibility was assessed by determining inter- and intra-assay variation with five replicates. Evaluation in mixed-culture systems was carried out by measuring target DNA recovered from rumen digesta which had a known amount of each bacterium added at three to four different quantities.

Enumeration of each bacterial species in various sheep digestive samples using the cPCR assays was carried out. Three sheep, fed twice daily, were used as sample donors. One sheep was fed on three different diets over 3 × 3-week periods. Diets consisted of alfalfa hay and commercial formula feed for beef cattle (200:800, 500:500 and 800:200 g/day). Whole rumen contents were withdrawn three times on each diet (days 19–21) through a ruminal fistula prior to morning feeding. The other two sheep, fed a hay (Sudan grass) and concentrate diet, were slaughtered and digesta from the rumen, omasum, abomasum, duodenum, ileum, caecum, colon and rectum obtained. Each sample was mixed and then sub-sampled (without separation of liquid from solid). DNA extraction from digestive samples was performed as described above, except abomasal and duodenal samples were neutralized with 1.0 M Tris-HCl (pH 8.0) prior to extraction. Extracted DNA preparations were all subjected to cPCR analysis.

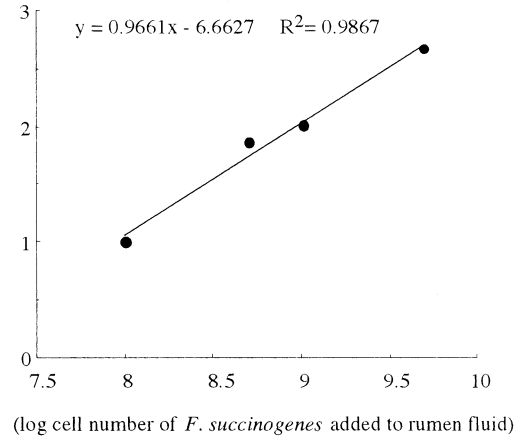
[A]

(a)

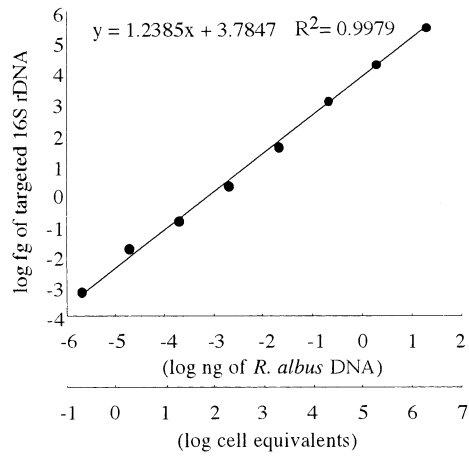


[B]

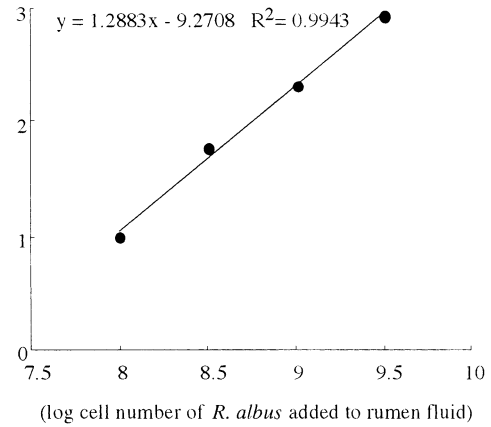
(d) (log fg of targeted 16S rDNA in 1ng of total ruminal DNA)



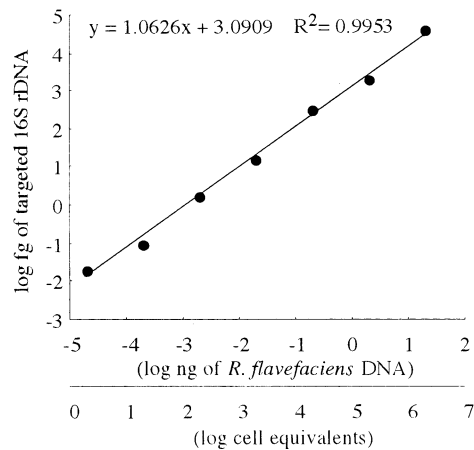
(b)



(e)



(c)



(f)

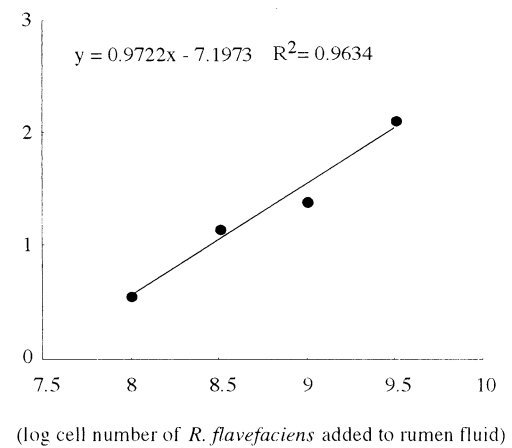


Fig. 1. Quantitative ability and sensitivity of cPCR assays at pure- (A) and mixed- (B) culture levels for *F. succinogenes* (a and d), *R. albus* (b and e) and *R. flavefaciens* (c and f). A: Relationship between DNA from pure culture and assay values of the targeted 16S rDNA; B: relationship between cells added to rumen fluid and assay values of the targeted 16S rDNA.

Table 1  
Specificity of PCR primers and tested bacterial strains

Bacterial species (strains)	Amplification by <sup>a</sup>			
	specific primers			general primers
	Fs	Ra	Rf	
<i>F. succinogenes</i> (S85, GC5)	+	–	–	+
<i>F. intestinalis</i> (LH1)	–	–	–	+
<i>R. albus</i> (7, F-40)	–	+	–	+
<i>R. flavefaciens</i> (FD-1)	–	–	+	+
<i>B. fibrisolvens</i> (D1, 49, GS113, OB156)	–	–	–	+
<i>Prevotella ruminicola</i> (23, 7-13)	–	–	–	+
<i>Ruminobacter amylophilus</i> (70)	–	–	–	+
<i>Succinivibrio dextrisolvens</i> (24)	–	–	–	+
<i>Succinimonas amylolytica</i> (B <sup>2</sup> 4)	–	–	–	+
<i>Streptococcus bovis</i> (NCDO597)	–	–	–	+
<i>Lactobacillus ruminis</i> (ATCC27780)	–	–	–	+
<i>Megasphaera elsdenii</i> (LC1)	–	–	–	+
<i>Eubacterium ruminantium</i> (GA195)	–	–	–	+
<i>Eubacterium cellulosolvens</i> (2)	–	–	–	+
<i>Selenomonas ruminantium</i> (ATCC12561)	–	–	–	+

+, amplified; –, not amplified

<sup>a</sup>Primers used are: Fs219f (5'-GGT ATG GGA TGA GCT T GC-3') and Fs654r (5'-GCC TGC CCC TGA ACT ATC-3') for *F. succinogenes*; Ra 1281f (5'-CCC TAA AAG CAG TCT TAG TTC G-3') and Ra 1439r (5'-CCT CCT TGC GGT TAG AAC A-3') for *R. albus*; Rf 154f (5'-TCT GGA AAC GGA TGG TA-3') and Rf 425r (5'-CCT TTA AGA CAG GAG TTT ACA A-3') for *R. flavefaciens*; FP27 (5'-AGA GTT TGA TCC TGG CTC AGG A-3') and Eub338r (5'-GCT GCC TCC CGT AGG AGT-3') for general (domain of bacteria). PCR conditions are same with those of cPCRs shown in the text.

### 3. Results and discussion

The primer pairs for each cellulolytic species did not amplify any of the 14 other rumen bacterial species tested, indicating the primer pairs were highly specific (Table 1). DNA from type strains of all three species successfully co-amplified with their respective constructed competitor DNA.

Amplification of templates composed of increasing competitor DNA, relative to target 16S rDNA, showed a highly linear relationship between the concentration of added competitor DNA to the amplified competitor/target DNA ratio (data not shown). To evaluate the quantitative ability and sensitivity of each cPCR assay, different amounts of DNA (cells) from each type strain were added. For all three cPCR assays, a positive response to the addition of different numbers of cells was observed (Fig. 1A). The lowest cell number plotted on the regression for each assay, 1–10 cells, indicates the minimum detection limit for each bacterial species in pure culture. Minimum detection limits in a mixed-culture system (rumen fluid plus pure culture) was not able to be clearly defined as all three cellulolytic species are indigenous to the rumen (Fig. 1B). As *R. albus* could be measured in sheep at  $10^4$  per ml rumen digesta (see below), and all three cellulolytic species were added to cell-free rumen fluid at a level of  $10^3$  per ml, the lowest detection limit of the assays in mixed-culture system was estimated to be  $10^{3-4}$  per ml. This limit, similar to minimal levels in other cPCR assays [8,9], allows the monitoring of rumen bacteria present at more than 0.00001% of total population ( $10^{10}$  per ml).

This suggests that developed cPCR assays are approximately 1000 times more sensitive than probing methods (0.01% minimum detection) and are adequate for assessing the cellulolytic bacteria functioning within the rumen.

Fig. 2 shows the population sizes of the target cellulolytics in the rumen and their responses to dietary change, as enumerated by the cPCR assays. *F. succinogenes* was most dominant ( $10^{6-7}$  per ml of rumen digesta) of the three species, followed by *R. flavefaciens* ( $10^5$  per ml) and *R. albus* ( $10^4$  per ml). Since the total size of the bacterial population in the rumen is around  $10^{10}$  per ml, our results suggest that *F. succinogenes* is present at only 0.1% of total population and ruminococci are quite minor. The scarcity of the two ruminococci is surprising considering they are considered representative cellulolytics, i.e. ruminal

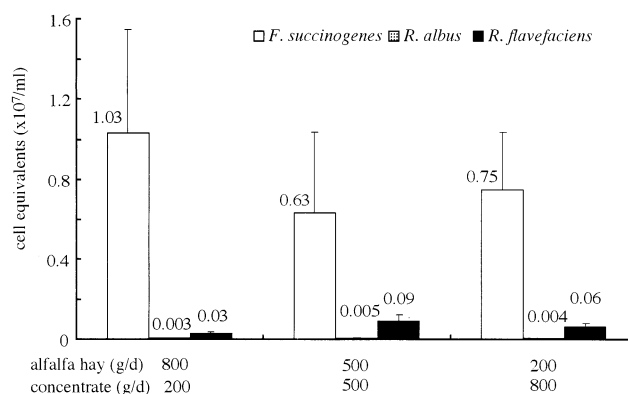


Fig. 2. cPCR-aided enumeration of the three representative cellulolytic bacterial species, *F. succinogenes*, *R. albus* and *R. flavefaciens*, in the rumen of a sheep fed three diets with different proportions of alfalfa hay.

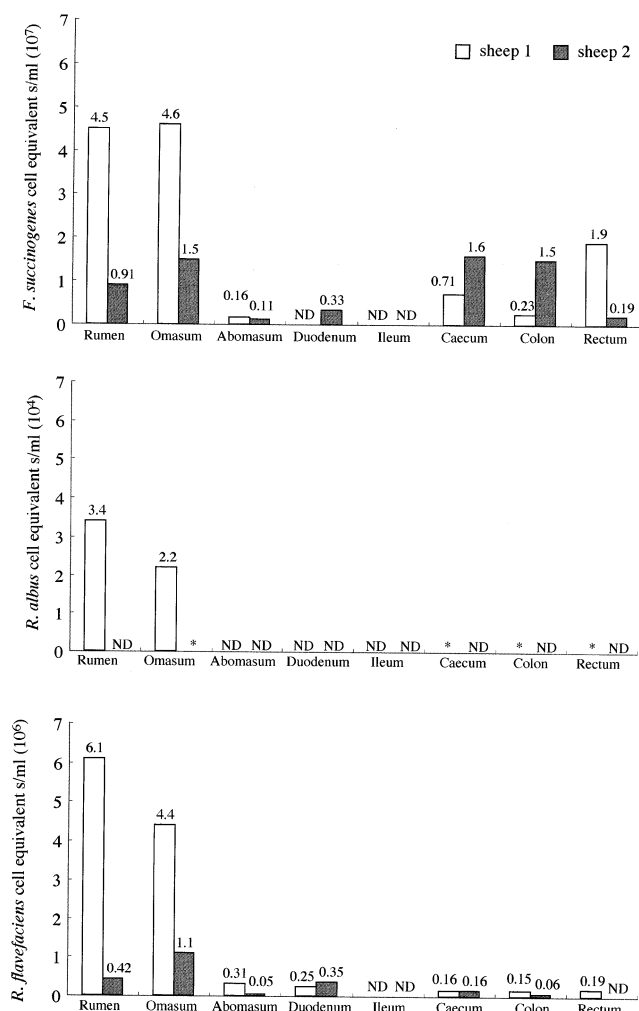


Fig. 3. cPCR-aided enumeration of the three representative cellulolytic bacterial species, *F. succinogenes*, *R. albus* and *R. flavefaciens*, in various alimentary tract sites of sheep on hay (Sudan grass) and concentrate diet. ND: not detected. \*: detectable but not quantifiable.

densities ranging from 0.1 [16] to 6.6% [17] for *F. succinogenes*, and from 1.3 to 2.9% for *Ruminococcus* spp. [18]. It is possible that dietary conditions may have lead to a reduced cellulolytic bacterial flora. Feeding of a 100% orchard grass hay diet increased these three cellulolytic species up to  $10^8$  per ml for *F. succinogenes*,  $10^7$  per ml for *R. flavefaciens* and  $10^5$  per ml for *R. albus* (Koike et al., unpublished results). Other cellulolytics residing in the rumen may be undescribed *Fibrobacter* and *Ruminococcus* [18], or perhaps novel species [19].

The proportion of roughage in the diet may influence the population size or the proportion of cellulolytic bacterial species in the rumen. However, the three cellulolytic species examined in the present study did not significantly respond to a change in proportion of alfalfa hay in the diet. Similar results were obtained in the rumen of dairy cows fed four different diets; no clear response to the diet change was observed in the rumen populations of the three cellulolytic species as determined by a DNA probe hybridization method [3].

The distribution of the three cellulolytic species in different sites of the sheep alimentary tract is shown in Fig. 3. As found in the rumen (Fig. 2), *F. succinogenes* was the most abundant of the three cellulolytic species, irrespective of alimentary tract site. Rumen and omasum were major habitats for all three cellulolytic species, and the hindgut (caecum, colon and rectum) also accommodated them. The distribution however depended on individual animals. High distribution of the cellulolytics in omasal digesta is reasonably explained by the fact that the omasal digesta mainly consists of plant fiber particles that are likely to have been colonized by the cellulolytics.

In conclusion, cPCR assays were newly established for three representative ruminal cellulolytic species: *F. succinogenes*, *R. albus* and *R. flavefaciens*. The assays showed high sensitivity and reproducibility, allowing enumeration of the three species from various sheep digestive sites in a quick and accurate manner. *F. succinogenes* was found to be the most dominant of the three species, with its ruminal distribution calculated to be 0.1% of the total bacterial population under the experimental dietary conditions.

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