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ORIGINAL ARTICLE

Quantification of ruminal *Clostridium proteoclasticum* by real-time PCR using a molecular beacon approach

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Abstract

Aims: All members of the ruminal *Butyrivibrio* group convert linoleic acid (*cis*-9,*cis*-12-18 : 2) *via* conjugated 18 : 2 metabolites (mainly *cis*-9,*trans*-11-18 : 2, conjugated linoleic acid) to vaccenic acid (*trans*-11-18 : 1), but only members of a small branch, which includes *Clostridium proteoclasticum*, of this heterogeneous group further reduce vaccenic acid to stearic acid (18 : 0, SA). The aims of this study were to develop a real-time polymerase chain reaction (PCR) assay that would detect and quantify these key SA producers and to use this method to detect diet-associated changes in their populations in ruminal digesta of lactating cows.

Methods and Results: The use of primers targeting the 16S rRNA gene of *Cl. proteoclasticum* was not sufficiently specific when only binding dyes were used for detection in real-time PCR. Their sequences were too similar to some non-producing strains. A molecular beacon probe was designed specifically to detect and quantify the 16S rRNA genes of the *Cl. proteoclasticum* subgroup. The probe was characterized by its melting curve and validated using five SA-producing and ten nonproducing *Butyrivibrio*-like strains and 13 other common ruminal bacteria. Analysis of ruminal digesta collected from dairy cows fed different proportions of starch and fibre indicated a *Cl. proteoclasticum* population of 2–9% of the eubacterial community. The influence of diet on numbers of these bacteria was less than variations between individual cows.

Conclusions: A molecular beacon approach in qPCR enables the detection of *Cl. proteoclasticum* in ruminal digesta. Their numbers are highly variable between individual animals.

Significance and Impact of the Study: SA producers are fundamental to the flow of polyunsaturated fatty acid and vaccenic acid from the rumen. The method described here enabled preliminary information to be obtained about the size of this population. Further application of the method to digesta samples from cows fed diets of more variable composition should enable us to understand how to control these bacteria in order to enhance the nutritional characteristics of ruminant-derived foods, including milk and beef.

Introduction

A high consumption of saturated fatty acids is generally accepted as a cardiovascular disease risk (Menotti *et al.* 1999) with evidence to suggest that high intakes of saturated fats may also reduce insulin sensitivity (Vessby *et al.* 2001). Milk and dairy products are typically the major source of saturated fatty acids in the human diet (Givens and Shingfield 2004). Therefore, there is interest in developing nutritional strategies for simultaneously enhancing

the concentrations of oleic (*cis*-9-18 : 1), linoleic (*cis*-9,*cis*-12-18 : 2, LA) and linolenic (18 : 3 n-3) acids and decreasing the proportions of lauric (12 : 0), myristic (14 : 0) and palmitic (16 : 0) acids in milk fat. Following ingestion, dietary unsaturated fatty acids are extensively metabolized in the rumen in a process generally referred to as biohydrogenation. Conversion of unsaturated to saturated fatty acid products is incomplete and a wide range of intermediates can accumulate (Harfoot and Hazlewood 1997; Palmquist *et al.* 2005). Ruminal metabolism of LA is known to proceed via rumenic acid (*cis*-9,*trans*-11-18 : 2, RA), which is sequentially reduced to form vaccenic acid (*trans*-11-18 : 1, VA). Therefore, ruminant fats also contain compounds, including RA, which have been shown to promote human health in several ways (Kritchevsky 2000; Whigham *et al.* 2000). Numerous experiments have shown that RA exerts anti-carcinogenic properties in cell culture and animal model studies and alter blood lipid profiles and immune function in humans (Wahle *et al.* 2004; Yaqoob *et al.* 2006), stimulating further interest in altering milk fatty acid composition for improved long-term human health. Understanding factors that regulate the final reduction of *trans* 18 : 1 intermediates to stearic acid (18 : 0, SA) is of major importance because VA acts as a substrate for endogenous RA synthesis in mammalian tissues via the action of stearoyl-CoA desaturase, whereas SA is converted by the same enzyme to oleic acid (Griinari *et al.* 2000; Palmquist *et al.* 2005). All this information highlights that ruminal biohydrogenation is fundamental to the supply of potentially beneficial fatty acids available for incorporation into ruminant milk and meat.

Biohydrogenation of unsaturated fatty acids is thought to be mainly because of strains from the *Butyrivibrio* group in the rumen (Polan *et al.* 1964; van de Vossenberg and Joblin 2003). The *Butyrivibrio* group, which includes the genus names *Butyrivibrio* and *Pseudobutyrivibrio* and a group named *Clostridium proteoclasticum* (Kopecny *et al.* 2003), comprises strains exhibiting large variation in their 16S rRNA sequences, and the taxonomy of this group of bacteria requires revision (Forster *et al.* 1996; Willems *et al.* 1996; van de Vossenberg and Joblin 2003). It has emerged recently (Wallace *et al.* 2006) that *Cl. proteoclasticum* corresponds to the bacteria named *Fusocillus* isolated as SA producers many years ago (Kemp *et al.* 1975). In a previous study (Paillard *et al.* 2007), a phylogenetic tree was constructed using 16S rRNA sequences of 47 ruminal strains of *Butyrivibrio*-like butyrate producers. Metabolic analysis of these strains revealed that 33 metabolized LA to conjugated linoleic acid or VA, whereas other closely phylogenetically related strains appeared to convert LA to SA.

Several methods for the detection and quantification of bacteria of the rumen by molecular techniques have been described based on competitive polymerase chain reaction (PCR; Reilly and Attwood 1998; Kobayashi *et al.* 2000; Koike and Kobayashi 2001), hybridization probes (Forster *et al.* 1997; Krause *et al.* 1999) or real-time PCR (Schofield *et al.* 1997; Tajima *et al.* 2001; Ouwerkerk *et al.* 2002; Mohammadi *et al.* 2003). These studies reported assays for the detection and enumeration of eubacteria (Mohammadi *et al.* 2003), *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* (Schofield *et al.* 1997; Krause *et al.* 1999; Koike and Kobayashi 2001), *Cl. proteoclasticum* (Reilly and Attwood 1998) or *Megasphaera elsdenii* (Ouwerkerk *et al.* 2002). Tajima *et al.* (2001) described 12 sets of primers for the detection and quantification of 13 ruminal bacteria other than *Butyrivibrio* spp. using a dye-binding approach. Competitive PCR methods for the enumeration of *Butyrivibrio fibrisolvens* (Mrazek and Kopecny 2001) or *Cl. proteoclasticum* and related strains (Reilly and Attwood 1998) have been developed, but in both studies the designed primers recognized bacteria that we now know do not produce stearic acid (Wallace *et al.* 2006). Thus far, there is no specific real-time PCR assay available for *Butyrivibrio*-like strains similar to *Cl. proteoclasticum* that have been shown to hydrogenate LA to SA.

In the present work, we describe the development and application of a real-time PCR method to detect and specifically quantify *Cl. proteoclasticum* in ruminal digesta. Samples of digesta were collected from a feeding study with lactating dairy cows fed diets of variable composition known to alter ruminal metabolism of unsaturated C18 fatty acids to SA (Palmquist *et al.* 2005). Dye-binding PCR methods lacked sufficient specificity and therefore a method based on the molecular beacon principle was developed. A unique feature of this technique involves exploiting variation in 16S rRNA genes, which has not been applied in previous molecular beacon studies.

Materials and methods

Strains and culture conditions

Butyrivibrio fibrisolvens D1^T (ATCC 19171^T) and *Butyrivibrio hungatei* DSM10295, *Pseudobutyrivibrio xylanivorans* DSM10296 and DSM10317, *Anaerovibrio lipolytica* 5S^T (ATCC 33276^T), *Clostridium aminophilum* F^T (ATCC 49906^T), *F. succinogenes* S85^T (ATCC19169^T), *M. elsdenii* LC1^T (ATCC 25940^T), *Methanobrevibacter smithii* PS (ATCC 35061), *Peptostreptococcus anaerobius* 4372^T (ATCC 27337^T) and *Prevotella ruminicola* 23^T (ATCC 19189^T) were obtained from culture collections. Strains *B. fibrisolvens* JK10/1, JK615^T (ATCC BAA-456^T), CE51, and Mz3, *B. hungatei* JK611 and JK614, *Cl. proteoclasticum*

JK618 and UC142, *Pseudobutyrivibrio ruminis* JK205 and Mz6, *P. xylanivorans* Mz4, Mz5^T (ATCC BAA-455^T), Mz7, Mz8, JK663, JK668, JK724, JK729 and JK730, and *Butyrivibrio* sp. OB156, Mz9, JK612, JK662 and JK684 were gifts from J. Kopečný, Prague, Czech Republic. Strains *B. fibrisolvens* O110 and *Butyrivibrio* sp. 25/2 were donated by K. Gregg, Perth, Western Australia. Strain *Lachnospira multipara* D15d was a gift from B.A. Dehority, Columbus, OH, USA and *Cl. proteoclasticum* C-proteo was received from G. Attwood, Palmerston North, New Zealand. *Prevotella bryantii* B_14^T (DSM 11371^T) was a gift from M. Bryant, Champaign, Illinois, USA. Strains *B. fibrisolvens* JW11 and SH13, *Cl. proteoclasticum* P-18, *R. albus* SY3, *R. flavefaciens* 17, *Selenomonas ruminantium* Z108, and *Streptococcus bovis* ES1 were isolated at the Rowett Research Institute, UK. All strains were cultivated in the liquid form of M2 medium (Hobson 1969), under CO_2 at 39°C.

Origin of ruminal digesta samples

Samples of ruminal digesta were obtained from an experiment with lactating dairy cows for which all experimental procedures were approved by the Experimental Animal Care Committee of MTT Agrifood Research, Finland. Four ruminally fistulated multiparous cows 71·5 ± 7·7 days in lactation were used in a 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments and 21-day experimental periods to evaluate the effect of level (forage : concentrate ratio 65 : 35 or 35 : 65 on a dry matter (DM) basis, L and H, respectively) and type (starch or fibre-rich, S and F, respectively) of concentrate in the diet of cows fed grass silage on ruminal lipid metabolism. Concentrate rich in starch comprised rolled barley, rolled oats, rapeseed expeller, vitamins and minerals. Fibre-rich concentrate was prepared by replacing barley and oats with barley feed and molassed sugar beet pulp. Samples of digesta were collected from four sites within the rumen-reticulum at 15:00 on day 17 and 09:00 on day 19 of each experimental period. Immediately after collection, 50 g subsamples were placed in liquid nitrogen and stored at -80°C. At the end of each experiment, subsamples collected during each period were composited within animal in order to simulate the average digesta composition in terms of location and time.

DNA extraction

Total genomic DNA from pure cultures was extracted using the DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Ruminal digesta samples were thawed and strained through two layers of muslin cloth and the rumen fluid obtained was submitted for analysis. Total genomic DNA was extracted as follows (Muetzel *et al.* 2006): 300 μl of ruminal fluid were mixed with 260 μl of STE buffer (10 mmol l^{-1} Tris-HCl pH 8·0, 100 mmol l^{-1} sodium chloride, 1 mmol l^{-1} EDTA pH 8·0), 40 μl of 20% SDS, and 0·5–1 g of 0·1 mm Zirkonia/Silica beads (Biospec Products, Bartlesville, OK, USA). Cells were lysed by three treatments of 30 s of bead beating, with 2 min intervals on ice, using the FastPrep® Instrument (Qbiogene, Cambridge, UK). Tubes were then centrifuged at 5000 ***g*** for 2 min. The supernatant was transferred to a fresh tube, extracted with phenol/chloroform/isoamylalcohol (25 : 24 : 1, v:v), and precipitated with 7·5 mol l^{-1} ammonium acetate and isopropanol. Nucleic acids were treated with DNase-free RNase (Promega). For further purification, the Fast Soil kit (Qbiogen) was used in accordance with the manufacturer's recommendations from the fifth step (following DNA extraction). This combination of methods maximized the recovery of DNA from ruminal digesta (Muetzel *et al.* 2006). The eluted DNA was dissolved in sterile buffer provided in the Fast Soil kit and stored at -20°C. DNA was quantified using the NanoDrop® ND-1000 Spectrophotometer (Labtech, Ringmer, UK).

Design of primers and probe

The primers applied in both approaches and the probe used in the present work were designed to detect the targeted strains of SA-producing bacteria from a group-specific region on the 16S rRNA gene (Table 1). The specific molecular probe was designed with a 23-nt loop sequence (Table 1, not underlined), and two sequences of five oligonucleotides (Table 1, underlined) formed the stem structure. Six fluorescein (FAM) and DABCYL were used as a fluorophore and quencher, respectively. All oligonucleotides were obtained from SIGMA-Genosys (Haverhill, UK). The specificity of primers and probe was confirmed *in silico* by cross referencing with the Ribosomal Database Project II website (URL: http://rdp.cme.msu.edu/index.jsp) (Cole *et al.* 2005). Theoretical hybridization temperatures and secondary structures of all oligonucleotides were checked using the Gene Runner programme (URL: http://www. generunner.com/).

Probe characterization

The signal-to-background ratio of the molecular beacon probe was calculated according to the following formula: $(F_{ch} - F_b)/(F_{uh} - F_b)$, where F_{uh} and F_{ch} represent the fluorescence intensity of unhybridized and completely hybridized probe, respectively, and F_b is the fluorescence intensity of buffer (Vet and Marras 2004). The efficiency

Table 1 Oligonucleotides designed to target the 16S rRNA gene of stearic acid-producing *Butyrivibrio*-related bacteria

Oligonucleotide name	Sequence (5′3′)
SA-20c*	GATTTGCTCCGGATCGCTCC
SA-11b*	CGGCTTACTGGACAGCAAC
SA–FW†	TCCGGTGGTATGAGATGGGC
SA–RV†	GTCGCTGCATCAGAGTTTCCT
Molecular beacon probe	FAM-CCGCTTGGCCGTCCGACCTCTCAGTCCGAGCGG-DABCYL

*Primers SA-20c and SA-11b were designed for the dye binding approach.
†Primers SA–FW and SA–RV were designed for the molecular beacon approach (see 'Materials and methods').

of quenching (E_{ff}) was determined by fluorescence analysis using the formula:

$$E_{ff} = \left(1 - \frac{F_{uh} - F_b}{F_{ch} - F_b}\right) \times 100$$

(Poddar 2000).

The melting curve experiment, or denaturation profile, was conducted by adding the probe to a final concentration of 200 nmol l^{-1} in a solution containing 10X buffer (Promega) and 3 mmol l^{-1} $MgCl_2$, with or without a five-fold molar excess of complementary DNA, in a total volume of 25 μl. The temperature was decreased from 95°C to 30°C in 1 C° increments, and at each temperature increment the fluorescence reading was allowed to stabilize for 1 min. All fluorescence measurements were made with the iCycler iQ thermal cycler (Bio-Rad, Hercules, USA).

Polymerase chain reaction

For the dye binding approach, various conditions of PCR were evaluated. Amplification was carried out in a final volume of 25 μl containing 50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl (pH 8·3), 1·5, 2, 2·5 or 3 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} (each) deoxyribonucleotide triphosphates, 1·5 U of Taq polymerase (Promega), 0·4 or 0·5 μmol l^{-1} (each) of primers SA-20c and SA-11b and 2·5 μl of purified genomic DNA. Amplification (10 min at 95°C, then 35 cycles of three steps consisting of 1 min at 95°C, 1 min at the hybridization temperature and 2 min at 72°C) was performed with the Mastercycler® Gradient (Eppendorf, Hamburg, Germany). PCR products were analysed on 2% agarose gels by electrophoresis.

Real-time polymerase chain reaction assay

Dilutions of purified genomic DNA from the control strain P-18 were used to construct specific calibration curves and assess the probe sensitivity. All samples were run in duplicate or triplicate (unless otherwise stated) in two independent experiments. Amplification was carried out in a final volume of 25 μl containing 12·5 μl of iQ Supermix (Bio-Rad), 400 and 800 nmol l^{-1} of primers SA–FW and SA–RV, respectively, 250 nmol l^{-1} of molecular beacon, and 2·5 μl of purified genomic DNA. Amplification (10 min at 95°C, then 40 cycles of three steps consisting of 30 s at 95°C, 1 min at 55°C and 30 s at 72°C) was performed with the iCycler iQ thermal cycler (Bio-Rad). Fluorescence data were collected at the end of the hybridization step, at an excitation and emission wavelength of 490 and 530 nm, respectively. Amplification of eubacterial DNA was carried out according to a previously published assay (Maeda *et al.* 2003). Results were analysed using the iCycler iQ detection system software (Bio-Rad). PCR products were analysed on 2% agarose gels by electrophoresis.

Statistical analysis

Regression analysis was used to fit standard calibration curves [Statistical Analysis Systems Institute (SAS) 2001]. Data concerning the application of the technique are expressed as the mean ± SE for three determinations. Measurements of *Cl. proteoclasticum* DNA as a percentage of total bacterial DNA determined in digesta samples from lactating cows were subjected to analysis of variance using the mixed linear model procedure of SAS. Data from samples collected from the feeding experiment were analysed according to a 4×4 Latin square with a 2×2 factorial arrangement of treatments with a model that included the random effects of cow and fixed effects of period, level of concentration, concentrate type and their interaction using the Satterthwaite option to calculate degrees of freedom for fixed effects (Littell *et al.* 1996). Means (± SE) for individual cows and least square means are reported with treatment effects being considered significant at $P < 0{\cdot}05$.

Results

Detection of members of the *Butyrivibrio* group by the dye-binding method

Sets of primers were designed to be used in real-time PCR using binding dyes. Primers SA-20c and SA-11b (Table 1)

were tested on four SA-producing *Cl. proteoclasticum* strains (P-18, JK205, JK668 and JK724) and 23 SA non-producing *Butyrivibrio*-like strains (25/2, CE51, DSM10295, DSM10296, DSM10317, JK611, JK612, JK614, JK618, JK662, JK663, JK684, JK729, JK730, Mz3, Mz4, Mz6, Mz7, Mz8, Mz9, O110, OB156 and SH13). Various PCR conditions were tested: four Mg^{2+} concentrations, two different primer concentrations, and a hybridization temperature ranging from 55°C to 73·2°C. Most of the samples produced at least one PCR product, even at the highest temperature tested, indicating a lack of specificity of the primers tested as well as amplification of undesirable products by mispairing. These primers were not tested in real-time PCR, as the binding molecules would have bound to both specific and nonspecific products.

Detection of members of the *Butyrivibrio* group by a molecular beacon-based method

Probe characterization

The possible occurrence of secondary structures was checked for the probe described in Table 1 using the Gene Runner programme. This analysis confirmed that the probe would have the correct conformation, i.e. stem-and-loop structure in its free form.

To evaluate the probe preparation quality and to discard chances of uncoupled fluorophores of partially/wrongly synthesized labelled oligonucleotides during the fabrication process, the signal-to-background ratio was first evaluated (Vet and Marras 2004), followed by measurements of the efficiency of quenching by fluorescence analysis (Poddar 2000). With our probe, the signal-to-background ratio was >20 and the efficiency of quenching of light emitted from the fluorophore was >95%. A melting curve was prepared to check the behaviour of the probe according to temperature. The fluorescence of our molecular beacon increased sigmoidally as temperature increased (Fig. 1). The T_m of the stem region was approx. 63°C (Fig. 1).

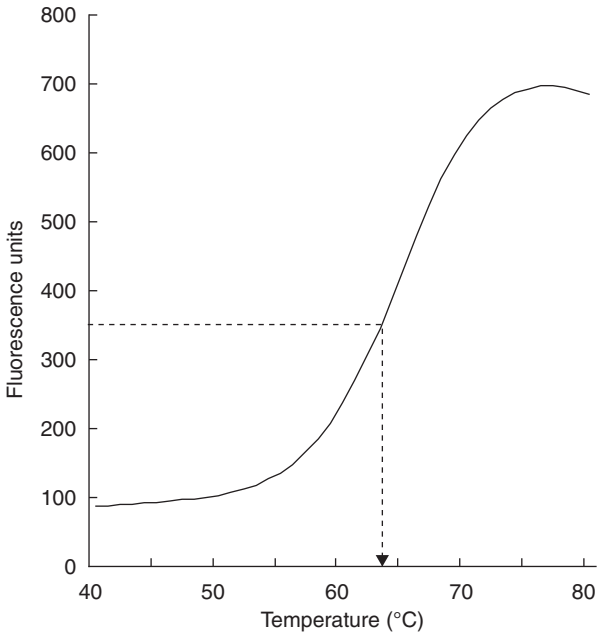

Figure 1 Thermal transition of the molecular beacon probe. The arrow indicates the T_m of the probe.

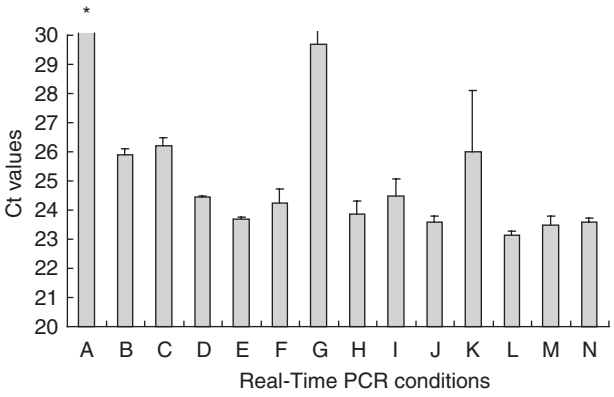

Figure 2 Variation in Ct values according to primer concentrations in real-time PCR. Concentrations tested (FW primer–RV primer, in nmol l^{-1}): A: 800–20, B: 600–100, C: 800–100, D: 600–200, E: 600–400, F: 800–400, G: 20–800, H: 100–600, I: 100–800, J: 200–600, K: 400–600, L: 400–800, M: 600–600, N: 800–800. Bars represent the standard errors of the mean based on four determinations. *Ct value >40.

Optimization of a real-time PCR assay based on a molecular beacon probe

The amplification efficiency of the real-time PCR assay was tested for various reagent concentrations and annealing temperatures to obtain the final parameters described in 'Materials and methods'. Several primer concentrations were tested using a fixed probe concentration (250 nmol l^{-1}). Assay sensitivity, as indicated by the Cycle Threshold (Ct) value varied markedly depending on primer concentrations (Fig. 2). Conditions that allowed for the most sensitive assay, resulting in the lowest Ct value (Fig. 2, condition L), were used in subsequent experiments. Three probe concentrations (250, 500 and 1000 nmol l^{-1}) were then tested with the optimal concentrations of primers determined previously (data not shown). The final assay comprised an asymmetric PCR (1 vol. SA–FW and 2 vol. SA–RV) and 250 nmol l^{-1} of probe.

Two samples of DNA extracted from ruminal digesta were tested in six replicates to determine the intra-assay precision under the defined optimal conditions described previously. Based on the Ct values, analysis of both

samples yielded mean ± SE values of 20·6 ± 0·129 and 24·5 ± 0·179, respectively (data not shown). The measured variance corresponds to an intra-assay coefficient of variation of 1·53 and 1·79%, respectively. Sensitivity tests using *Cl. proteoclasticum* P-18 DNA dilutions demonstrated that the assay efficiently detected the 16S rRNA gene in strains of the SA-producing group with a detection limit of 0·072 pg DNA (Ct value of 33). Standard curves of Ct against DNA concentration were constructed using serial dilutions of genomic DNA from strain P-18. The fitted curve exhibited linearity over a range of 5 $\log_{10}$, with an overall mean efficiency of 96·8% (slope: -3·399) and a correlation coefficient of 0·996 (results not shown). Similar results were obtained from the other SA-producing strains.

The specificity of the molecular beacon probe was checked on the Ribosomal Database Project II website (Cole *et al.* 2005). No complementary matches were found amongst ruminal nontarget organisms. The specificity of the probe was also determined experimentally by real-time PCR using five SA-producing *Cl. proteoclasticum* isolates (strains P-18, C-proteo, JK205, JK724 and UC142), 10 SA nonproducing isolates (strains Mz5^T, Mz8, JW11, JK10/1, JK612, JK614, JK615^T, JK618, JK684 and D1^T), and 13 other bacterial species common in the rumen (Table 2). The amount of DNA used as a template was standardized to 25 ng for each bacterium. A sample was interpreted as positive if the relative fluorescence crossed the threshold as determined by the iCycler detection system software. The 16S rRNA gene of the five SA-producing strains was detected by the assay, with a consistent Ct value being between 15·5 and 21·0, depending on the strain. The 23 remaining strains were not detected despite the presence of a PCR product in most cases (Table 2). Fluorescence emission was barely detectable (at least 92% decrease) when our probe was hybridized to a fivefold molar excess of a synthetic target designed to contain two mismatches in the central part of the oligonucleotide (data not shown). All these results provide clear evidence that the beacon is highly discriminatory, and is capable of selectively detecting DNA from *Cl. proteoclasticum* strains involved in the synthesis of SA as originally intended.

Table 2 Characterization of bacterial strains and response in a real-time PCR assay based on the molecular beacon approach

Species (strain no.)	PCR product*	Fluorescence†
Clostridium proteoclasticum (P-18)	+	+
Cl. proteoclasticum (C-proteo)	+	+
Pseudobutyrivibrio ruminis (JK205)	+	+
Cl. proteoclasticum (UC142)	+	+
Pseudobutyrivibrio xylanivorans (JK724)	+	+
B. fibrisolvens (D1^T)	–	–
Butyrivibrio fibrisolvens (JW11) (Wallace and Brammall 1985)	–	–
Butyrivibrio hungatei (JK615^T)	+	–
P. xylanivorans (Mz5^T)	+	–
Butyrivibrio sp. (JK612)	+	–
P. xylanivorans (Mz8)	+	–
B. hungatei (JK614)	+	–
Butyrivibrio sp. (JK684)	–	–
Cl. proteoclasticum (JK618)	+	–
Butyrivibrio sp. (JK10/1)	+	–
Anaerovibrio lipolytica (5S^T)	+	–
Clostridium aminophilum (F^T)	+	–
Fibrobacter succinogenes (S85^T)	+	–
Lachnospira multipara (D15d)	+	–
Megasphaera elsdenii (LC1^T)	+	–
Methanobrevibacter smithii (PS)	+	–
Peptostreptococcus anaerobius (4372^T)	+	–
Prevotella bryantii (B$_1$ 4^T)	+	–
Prevotella ruminicola (23 ^{T})	+	–
Ruminococcus albus (SY3)	+	–
Ruminococcus flavefaciens (17)	–	–
Selenomonas ruminantium (Z108)	+	–
Streptococcus bovis (ES1)	+	–

*Indicates the detection (+) or absence (–) of PCR product on a 2% agarose gel.

†Indicates the detection of fluorescence (+) or no fluorescence (–) in the same experiment.

Application of the molecular beacon approach to the analysis of ruminal digesta

Incremental addition of *Cl. proteoclasticum* P-18 DNA to a sample of bovine ruminal digesta DNA which contained low numbers of *Cl. proteoclasticum* DNA produced a linear response (results not shown). The slope of the fitted curve was -3·400 ± 0·019, consistent with that of the standard calibration curve described above (-3·399 ± 0·156).

This novel technique was applied to the analysis of samples of ruminal digesta collected from a feeding trial with lactating dairy cows offered diets containing different amounts of starch and fibre. DNA was extracted from 15 samples using a phenol–chloroform-based technique (Muetzel *et al.* 2006) and used in the optimized real-time PCR assay for *Cl. proteoclasticum*. DNA from *Cl. proteoclasticum* accounted for between 2·2 ± 0·1% and 9·4 ± 0·6% of total eubacterial DNA (Table 3). The variation was not related to treatment. Increases in the amount or type of concentrate had no effect on the relative abundance of *Cl. proteoclasticum* in ruminal digesta (Table 3).

Discussion

There are two main approaches to detect DNA amplification in real-time PCR experiments: using binding dyes

Table 3 Effect of level and type of concentrate in the diet on the relative populations of *Clostridium proteoclasticum* in ruminal digesta of cows fed total mixed rations based on grass silage

	Low-forage diet†		High-forage diet†			*P**		
Cow	Fibre	Starch	Fibre	Starch	SE	L	T	L × T
Kielo	7·0 ± 0·4	8·3 ± 1·2	3·6 ± 0·1	5·1 ± 0·5				
Leima	6·5 ± 0·9	2·4 ± 0·3	5·1 ± 0·2	2·8 ± 0·4				
Opus	9·4 ±0·6	2·2 ± 0·1	3·7 ± 0·5	–				
Osinko	8·1 ± 1·2	3·6 ± 0·2	7·0 ± 1·0	7·8 ± 1·4				
Average	7·8	4·1	4·9	5·2	1·11	0·35	0·16	0·22

Values represent the mean amount of *Cl. proteoclasticum* DNA as a percentage of total bacterial DNA based on three determinations.

*Probability of significant effects due to the level (L) or type (T) of concentrate in the diet, and their interaction (L × T).

†Grass silage-based diets were formulated to contain 350 (low) of 650 (high) g forage kg^{-1} dry matter supplemented with fibre or starch-rich concentrates.

that are nonspecific molecules that intercalate in the newly synthesized DNA, or hybridization probes, which are highly specific as they bind to an exact complementary target DNA. In the first approach, the primers have to be highly specific as to avoid synthesis of undesirable PCR products. The second approach uses a set of primers for amplification of a DNA fragment, and in addition, a highly specific probe labelled with a fluorogenic substance that is able to pair with the nascent DNA fragment emitting a signal upon interaction. The overall specificity of the PCR reaction is thus substantially enhanced.

In our first approach, sets of primers designed to detect the *Butyrivibrio* group were tested in PCR but revealed a lack of specificity, such that real-time PCR would lead to an overestimation of these bacteria in most of the samples. In certain cases, it is commonly known that oligonucleotides can prime DNA when the target sequence differs slightly from the original sequence from which the primer was designed; thereby enabling nonspecific PCR products to be formed. Here, after multiple sequence alignment analysis, no other region was found in the 16S rRNA genes of *Butyrivibrio*-like bacteria that could be used for primer design to allow different subgroups to be distinguished *via* qPCR using a dye-binding approach. Therefore, a combined primer-probe strategy was investigated.

Several kinds of probes have been described in the literature and extensive studies have demonstrated that molecular probes are more specific than binding dyes. Ouwerkerk *et al.* (2002) reported a Taq nuclease assay to enumerate *M. elsdenii* in ruminal contents, using an oligonucleotide probe which is hydrolysed by the exonuclease activity of the Taq polymerase, leading to the release of the fluorescence label and emission occurring during the extension phase of the PCR cycle. Molecular beacon probes have also been reported to be much more specific than binding dyes (Tyagi and Kramer 1996; Vet and Marras 2004). These probes are designed to contain a short complementary sequence of nucleotides at each end so that a stem-and-loop structure forms in solution (Tyagi and Kramer 1996). A fluorophore and a suitable quencher are attached *via* linkers to the stem ends so there is no fluorescence emission when the probe is in a closed configuration. Once hybridized to its complementary target during the annealing phase of the PCR cycle, the conformation of the probe is altered leading to a linear structure, allowing the quencher and fluorophore to become separated, leading to fluorescence emission (Tyagi and Kramer 1996). Development of real-time PCR assays based on a molecular beacon is comprised of two main steps: (i) probe characterization, involving checks of probe quality and denaturation profile; (ii) assay optimization, that requires assay calibration using standard curves and an assessment of assay reproducibility, sensitivity and specificity in a variety of controlled experiments.

Molecular beacon synthesis and purification are crucial steps for the production of the high quality probes that are essential for accurate and reliable experiments (Vet and Marras 2004). The presence of uncoupled fluorophores or oligonucleotides containing a fluorophore but lacking a quencher results in a low signal-to-background ratio and an erroneous fluorescence signal during real-time PCR (Vet and Marras 2004). Therefore, an evaluation of the probe preparation quality is necessary, by calculating the signal from the standard to that of the background. Our values of efficiency of quenching (>95%) and signal-to-background ratio (>20) are indicative that our molecular probe is of high quality and can be used for highly specific qPCR experiments in accordance with published outlines (Vet and Marras 2004). The second essential step for probe characterization is checking the probe behaviour according to temperature, or melting curve. In this experiment, minimum fluorescence indicates that the probe is 'closed', i.e. the quencher and fluorophore are located in close proximity. Maximum fluorescence indicates that the probe conformation has

become disordered with the quencher being sited relatively far away from the fluorophore, allowing emission from the fluorophore to be detected (Tyagi and Kramer 1996; Vet and Marras 2004). Fluorescence detection at low temperature means that there is a high background of fluorescence during experiments. The pattern obtained with our probe was similar to the one obtained in previous studies (Tyagi and Kramer 1996; Schofield *et al.* 1997; Poddar 2003). Fluorescence should be measured at a lower temperature (55°C in the present work) than the hybridization temperature of the stem region so that the nonhybridized probes are in the closed position to minimize overestimation of fluorescence. For most studies, it is difficult to establish if the assays used have been designed in this way, as the T_m of the stem region of the probe is rarely mentioned.

In this study, the location of the probe in the 16S rRNA gene aided by a multiple alignment analysis was crucial so it was developed before the primers. New primers were designed (Table 1), as the primers used in the first approach (dye-binding approach) were not located on either side of the probe. The size of the amplicon was 176 bp, which is close to the size of PCR amplicons detected by probes in previously published studies (Poddar 2000; Ouwerkerk *et al.* 2002; Poddar 2003). Amplicons with a larger size (as large as 800 bp) have been described in real-time PCR assays using binding dyes (Tajima *et al.* 2001), or in competitive PCR assays (Reilly and Attwood 1998). However, it is possible that molecular beacon-based detection is more efficient when the amplicon is relatively short, typically less than 200 bp (Poddar 2000), although there are no reported data in the literature to sustain this claim.

In real-time PCR assays involving fluorescent probes, the detection sensitivity depends on the amount of probe that hybridizes to its target strand. This, in turn, depends on the amount of available single-stranded target and the competition between the strand complementary to the target and the probe. The use of an asymmetric PCR rather than a symmetric PCR will generate more copies of the target strand in relation to the complementary strand, minimizing competition with the probe and thus improving the subsequent signal emission (Poddar 2000). Our data showed that the assay developed, based on an asymmetric PCR, is precise and highly reproducible, but it is difficult to compare these data with the literature, as intra-assay variation is rarely reported. In a competitive PCR optimized for the detection and quantification of *Cl. proteoclasticum* and related strains in ruminal samples, a comparable detection limit to the one obtained with our probe (0·072 pg DNA) was reported (Reilly and Attwood 1998), with a designed primer recognizing DNA from both what we now know to be VA- and SA-producing bacteria (Wallace *et al.* 2006; Paillard *et al.* 2007). Using the standard curve, it is possible to quantify the number of gene copies or bacteria present in the starting material (Ouwerkerk *et al.* 2002), providing that the number of copies of the gene in the target genome is known. The number of the 16S rRNA operon(s) and the size of the whole genome of strains of the *Butyrivibrio* group have not yet been determined. Therefore, the results in the present work are expressed as Ct values or relative DNA concentration, in common with most studies.

Our experiments indicated that the probe recognized only DNA from SA-producing strains of *Cl. proteoclasticum* (five tested) and not DNA from SA nonproducing *Butyrivibrio* strains (23 tested), demonstrating its high specificity. A decrease of at least 92% in fluorescence was detected when our probe was hybridized to a mismatched target. Schofield *et al.* (1997) noted a decrease of 80% in fluorescence using probes detecting *R. albus* or *F. succinogenes* when hybridized to a synthetic probe containing mismatches, while Tyagi and Kramer (1996) reported that there was no fluorescence in the presence of a 1-base mismatched oligonucleotide target.

Incremental addition of *Cl. proteoclasticum* DNA to a sample of bovine ruminal digesta DNA indicated that amplification and quantification of DNA would be equally specific for samples containing only target DNA and samples containing large amounts of nontarget DNA.

This novel technique was applied to the analysis of samples of ruminal digesta collected from an experiment with lactating dairy cows offered a diet containing different amounts of starch and fibre that were expected to alter ruminal lipid metabolism based on earlier studies by Palmquist *et al.* (2005). The relative size of the *Cl. proteoclasticum* population ranged from $2{\cdot}2 \pm 0{\cdot}1\%$ to $9{\cdot}4 \pm 0{\cdot}6\%$ of total eubacterial DNA. This proportion is consistent with the occurrence of these strains in isolation experiments (Kemp *et al.* 1975; van de Vossenberg and Joblin 2003; Wallace *et al.* 2006). In terms of numbers, Reilly and Attwood (1998) reported $2{\cdot}01 \times 10^6$ to $3{\cdot}12 \times$ *Cl. proteoclasticum* and related bacteria per ml of rumen fluid. If the total number of ruminal bacteria was about 10^{10} cells per ml, this would represent *Cl. proteoclasticum* at a maximum of 0·3% of the population, much lower than the proportion detected here. Both analyses depended on determining the abundance of the 16S rRNA gene. This leads to uncertainty because the number of 16S rRNA genes in *Cl. proteoclasticum* is not known, but it would not explain the different proportions in the different studies. Different diets often explain differences like this. In both our studies and those of Reilly and Attwood (1998), a range of dietary composition in terms of nitrogen and energy was used. The difference was in the basal diet, which was grass silage in the present study and

ryegrass pasture in Reilly and Attwood (1998). It is possible that fresh forage, with its readily available polyunsaturated fatty acids – which are highly toxic to *Cl. proteoclasticum* (Wallace *et al.* 2006) – leads to a lower *Cl. proteoclasticum* population than grass silage. Whatever the reason, both studies demonstrated a high variation between different animals in the size of the *Cl. proteoclasticum* population.

Earlier studies showed that lowering the proportion of forage in total mixed rations from 0·7 to 0·4 of diet DM decreased the reduction of *trans*-18 : 1 to SA in the rumen and altered the profile of biohydrogenation intermediates at the duodenum in lactating dairy cows (Palmquist *et al.* 2005). The animal experiment was carried out here in order to achieve this difference in fatty acid metabolism and to investigate any corresponding change in the *Cl. proteoclasticum* population. Increases in the amount or type of concentrate in the diet had no effect on the apparent relative abundance of *Cl. proteoclasticum*, however. Parallel analyses of the flows of SA or total *trans*-18 : 1 at the omasum were consistent with the dietary manipulation having failed to induce any change in fatty acid metabolism (Shingfield *et al.*, unpublished). Thus, further experiments will have to be conducted in order to obtain a shift in biohydrogenation and then to correlate population with the extent of biohydrogenation of LA to SA. At present, the *Cl. proteoclasticum* group are the only bacteria that have been isolated from the rumen which form SA (Kemp *et al.* 1975), but it is conceivable that other species, which are unculturable or have yet to be cultivated *in vitro*, may also carry out biohydrogenation. Direct detection of biohydrogenating ruminal bacteria is not yet possible. The gene that forms *trans*-10, *cis*-12-18 : 2 has been identified in *Propionibacterium acnes* and the protein characterized (Liavonchanka *et al.* 2006), but the genes encoding the enzymes that carry out the isomerization of LA to *cis*-9,*trans*-11-18 : 2 and its subsequent reduction have yet to be identified.

Conclusion

Application of the technique described here for the analysis of bacterial populations in ruminal digesta represents an effective mean of using real-time PCR with a molecular beacon to detect and selectively discriminate between *Butyrivibrio*-related bacteria that produce SA from those that do not, where sequence differences between strains are too small to use a real-time PCR based solely on dye-binding. The assay developed was shown to be precise, reproducible, sensitive and highly specific, and it should be useful in future studies aimed at linking the bacterial community in the rumen with the fatty acid composition of ruminant products. The principle may also be useful when applied to other groups of bacteria which are phylogenetically similar but have markedly different metabolic activities.

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