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Development of a RT-qPCR method for the quantification of *Fibrobacter succinogenes* S85 glycoside hydrolase transcripts in the rumen content of gnotobiotic and conventional sheep

Christel Béra-Maillet *, Pascale Mosoni, Anthony Kwasiborski, Florent Suau, Yves Ribot, Evelyne Forano

INRA, UR454 Unité de Microbiologie, F-63122 Saint-Genès-Champanelle, France

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ABSTRACT

An improved RNA isolation method based on the acid guanidinium–phenol–chloroform (AGPC) procedure using saline precipitation but no column purification was evaluated for quantifying microbial gene expression using reverse transcription quantitative PCR (RT-qPCR) in rumen contents.
The method provided good RNA integrity and quantity extracts. The transcript levels of eight glycoside hydrolase (GH) genes of the major rumen fibrolytic bacterium *Fibrobacter succinogenes* were quantified in the complex microbiota of a conventional sheep and in a gnotobiotic lamb harboring a microflora containing *F. succinogenes* S85 as the sole cellulolytic microorganism. This study validated the improved RNA isolation method, RT-qPCR conditions to quantify GH transcripts using either the *F. succinogenes* S85 *tuf* gene or the 16S rRNA-encoding gene (*rrs*) as the reference gene, and demonstrated the need to work with good quality RNAs. Transcripts from all the selected genes *cel3*, $endA_{FS}$, *celF* and *endB* endoglucanase genes, *cedA* cellodextrinase gene, *mlg* lichenase gene, and *xynC* and *xynD* xylanase genes of *F. succinogenes* S85 were detected and quantified at varying levels in the rumen content of the two animal models. This study opens new perspectives in studying microbial gene expression in the rumen of both conventional and gnotobiotic sheep.

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1. Introduction

The degradation of plant cell walls by ruminants is of major economic importance and is unique in that it relies on the cooperation between microorganisms that produce fibrolytic enzymes and the host animal that provides an anaerobic fermentation chamber. Increasing the efficiency with which the rumen microbiota degrades fiber has been the subject of extensive research but knowledge on how the major fibrolytic bacteria and fungi behave in the rumen is still lacking (Krause et al., 2003). The recent advances in molecular biology techniques have rendered the study of microbial functions and their interaction with other organisms and their environment in natural microbial habitats possible. Some of these methods, such as the metagenomic approaches, use DNA isolation from complex microbial ecosystems to create libraries of environmental DNA (Rodriguez-Valera, 2004; Warnecke et al., 2007) while others use RNA isolation to study microbial gene expression in complex environments such as soil (Saleh-Lakha et al., 2005). Though metagenomic approaches have already been used in the rumen and have unraveled the presence of novel polyphenol oxidase and extended hydrolase diversity (Ferrer et al., 2005; Galbraith et al., 2004; Krause et al., 2003), no studies on microbial gene expression have yet been reported in this ecosystem. Once the genomes of the rumen fibrolytic bacteria such as *Ruminococcus flavefaciens* FD1, *Fibrobacter succinogenes* S85, *Ruminococcus albus* 8, and *Prevotella ruminicola* 23 (Morrison et al., 2003) will be completely sequenced and annotated, the development of whole genome microarrays will probably be a powerful tool to examine gene expression by these organisms in vivo.

The present study aimed at optimizing total RNA isolation from rumen contents (RC) and finding RT-qPCR conditions to quantify GH transcripts and measure the in vivo expression of eight target glycosyl hydrolases (GH) genes of *F. succinogenes* S85 using reverse transcription quantitative PCR (RT-qPCR), knowing that such techniques will later be useful and complementary to future microarray approaches. *F. succinogenes* was chosen because it is a major fibrolytic bacterium within the rumen (Mosoni et al., 2007). It is particularly efficient in degrading various forms of crystalline cellulose and it shows a high ability to solubilize different plant cell wall polysaccharides (Forsberg et al., 2000). The enzymatic system of *F. succinogenes* S85 has been extensively studied by molecular and biochemical approaches, and about 20 enzymes have been characterized in vitro (i.e. many different cellulases, xylanases, ferulic acid and acetylxylan esterases, alpha-arabinofuranosidase, and alpha-glucuronidase exist in this organism)

* Corresponding author. Mailing address: INRA, UR454 Unité de Microbiologie, CR de Clermont-Ferrand/Theix, F-63122 Saint-Genès-Champanelle, France. Tel.: +33 473 62 40 67; fax: +33 473 62 45 81.
E-mail address: cmaillet@clermont.inra.fr (C. Béra-Maillet).

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(Forsberg et al., 2000). More recently, preliminary data obtained from the genome sequence of strain S85 tend to show that the fibrolytic system of this bacterium is much more complex than previously thought since more than one hundred genes encoding enzymes potentially active against carbohydrates have been identified (Qi et al., 2005). Furthermore, little is known about the concurrent mode of action of the enzymes on natural substrates and how their expression is regulated. We previously studied the effect of different carbon sources such as sugars, soluble purified polysaccharides (β-glucan, lichenan) or intact plant polysaccharides on the endoglucanase activity of *F. succinogenes* S85 and our results suggested an induction of endoglucanases by insoluble substrates (Béra-Maillet et al., 2000). In addition, the transcript level of 10 glycoside hydrolase (GH) genes (i.e. one xylanase, one lichenase and eight cellulase genes) was quantified in in vitro cultures of *F. succinogenes* grown on glucose, cellobiose or cellulose. The results showed that all the genes were transcribed, whatever the carbon source, and were over-expressed in cultures grown on cellulose (Béra-Maillet et al., 2000). In a recent study using NMR, the nature of the compounds released during in vitro degradation of wheat straw by *F. succinogenes* suggested a coordinate activity of xylanases and esterases on plant cell walls (Matulova et al., 2005). Despite all the data accumulated on the fibrolytic system of this bacterium, the way by which the bacterium regulates the expression of each GH gene under in vivo conditions so that its enzymatic system is optimal to degrade plant cell walls is still unknown. In order to progress in the understanding of how *F. succinogenes* acts in vivo, the expression of its GH genes must be quantified in the RC of ruminants in response to different biotic and abiotic stimuli. For this purpose, conditions were set up to validate an accurate quantification method of selected GH transcripts of *F. succinogenes* S85 in the RC of a sheep. We optimized RNA isolation from rumen contents and determined RT-qPCR conditions to estimate the transcript abundance of eight GH genes either in a conventional sheep which rumen microbiota is complex or in a gnotobiotic lamb harboring a diversified rumen microflora containing *F. succinogenes* S85 as the sole cellulolytic microorganism (Fonty et al., 1989) but no archaea, fungi and protozoa.

2. Materials and methods

2.1. Animals and rumen sampling

An INRA 401 gnotobiotically-reared lamb was obtained as previously described (Chaucheyras-Durand and Fonty, 2001) except that the lamb was placed in a sterile isolator 15 h after birth. Under these conditions, the rumen is colonized by a diversified microflora but cellulolytic bacteria, fungi, protozoa and methanogenic archaea do not establish (Fonty et al., 1987, 1988). The lamb can then be inoculated with selected microorganisms. In this experiment, the absence of cellulolytic microorganisms was verified using cultural method before the lamb was inoculated twice with pure cultures of the cellulolytic bacterium *F. succinogenes* S85 as described by Fonty et al. (1989). The absence of methanogens, fungi and protozoa was also verified. The lamb was fed ultrahigh-temperature-sterilized cow milk until the weaning and it also received sterile water ad libitum. During the experimentation, the animal received 1 kg per day of dehydrated lucerne hay (7-mm pellets sterilized by γ irradiation) distributed in the morning. A conventional Texel sheep was reared with meadow hay twice a day at maintenance level, with water ad libitum. It was cannulated as previously described (Brossard et al., 2004). Both animal experimentations received approval from the local ethic committee.

Sample (400 ml) of mixed liquid/solid rumen content (50:50) was collected via the permanent rumen cannula from the conventional sheep 6 h after feeding. Rumen fluid (approximately 20 ml) of the gnotobiotic lamb was withdrawn before the morning feed using a stomach flexible plastic tube. Immediately after withdrawal, rumen samples of conventional and gnotobiotic animals were mixed with two volumes of RNAprotect bacteria reagent (Qiagen, Courtaboeuf, France), aliquoted and stored at −80 °C.

2.2. Bacterial strain, growth conditions and enumeration

F. succinogenes S85 (ATCC 19169) was grown on a medium containing 40% rumen fluid and 0.3% cellobiose as previously described (Béra-Maillet et al., 2000). The enumeration of cellulolytic bacteria in RC was performed using the MPN method with McGrady's tables (Clarke and Owens, 1983).

2.3. DNA isolation

Genomic DNA was isolated from a 15-h culture of *F. succinogenes* S85 using the Easy-DNA kit from Invitrogen (Cergy Pontoise, France), according to the manufacturer's recommendations.

2.4. RNA isolation and reverse transcription

Total RNA was first isolated using a method hereafter named “Control method” based on the acid guanidinium–phenol–chloroform (AGPC) procedure (Chomczynski and Sacchi, 1987). Briefly, RNA-protected rumen content (12 ml) was defrosted and centrifuged for 20 min at 11,000 g and 4 °C. The pellet was resuspended in 12 ml of a RNA-E solution containing solution D from Chomczynski and Sacchi (1987), water saturated phenol and sodium acetate 0.2 M pH 4.0 (1:0.1:1). Cells were then disrupted by bead beating for 2 min with 0.1 g zirconia beads (0.1 mm) followed by a 2-min incubation at 60 °C. These two steps were then repeated. After addition of 4.5 ml of chloroform, the sample was briefly mixed, incubated for 15 min on ice and centrifuged (12,000 g, 20 min, 4 °C). The RNA contained in the aqueous supernatant (approximately 8 ml) was precipitated with 0.25 volume isopropanol and washed with 1 volume 75% cold ethanol in DEPC-treated water. Total RNA was solubilized in 700 µl of DEPC-treated water, treated with 10 U FPLC-pure DNAse I (G.E. Healthcare, Orsay, France) for 45 min at 37 °C, purified and concentrated with affinity column (UltraClean™ Microbial RNA isolation kit, Mo Bio Laboratories distributed by Ozyme, Saint-Quentin-en-Yvelines, France), and stored at −80 °C.

In order to optimize RNA isolation, several modifications of the control method were tested: 4-fold more bead quantity; no heat shock at 60 °C; 5-fold more RNA-E volume; RNA precipitation with 0.25 volume isopropanol and 0.25 volume saline solution (1.2 M NaCl, 0.8 M disodium citrate); no purification with affinity column. In order to compare the different extraction methods, they were performed in duplicate using aliquots of the same RC sample from the conventional animal.

RNA purity and concentration were estimated by measuring absorbance at 260 and 280 nm. RNA concentration and integrity were estimated using an Agilent 2100 Bioanalyser. The RNA 6000 Nanolabchip kit (Agilent Technologies, Massy, France) and the RNA 6000 ladder (Ambion, Applied Biosystems, Courtaboeuf, France) were used according to the manufacturer's recommendations. The 23S/16S rRNA peak area ratio and the RNA Integrity Number (RIN) analyses (Hawtin et al., 2005) were performed for each RNA sample using the 2100 expert software version B02-03 (Agilent Technologies, Massy, France).

Total RNA (1 µg) was reverse-transcribed into cDNA using random hexamer primers (Invitrogen, Cergy Pontoise, France) and 200 U SuperscriptII Rnase H^{-} reverse transcriptase (Invitrogen) according to the procedure supplied with the enzyme. For each RNA sample, a negative RT (no addition of reverse transcriptase) was performed and used as a negative control in subsequent PCRs.

GH transcript quantifications were performed using RNA isolated with the retained optimized procedure. Two RNA extracts (Conv 1 and Conv2) obtained from the RC of the conventional sheep and two RNA

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