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Occurrence of restriction–modification systems in ruminal butyrate-producing bacteria

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Abstract

Thirty-five strains of ruminal bacteria belonging to the former *Butyrivibrio fibrisolvens* species were screened for the presence of site-specific restriction endonuclease and modification methyltransferase activities. Seven strains possessed endonuclease activities detectable in crude cell extracts. The recognition sequences and optimal reaction conditions for seven of them were determined. Five enzymes were found to be isoschizomers of type II endonucleases (EcoRV, NsiI, AseI (2*x*) and SauI), one was type IIS (FokI) and two remained unknown. The optimal reaction buffer was found to be a low ionic strength buffer and all enzymes possessed sufficient activity at 39 °C. The presence of DNA modification among all strains was also determined. Most of the methylation activities correlated with restriction activities, yet some strains possessed unaccompanied modification methyltransferases.

Keywords: Restriction endonuclease; *Butyrivibrio fibrisolvens*; DNA modification

1. Introduction

Motile and butyrate-producing anaerobic bacteria are one of the most abundant bacterial groups in the rumen, and are represented mainly by *Butyrivibrio fibrisolvens* strains [1]. They belong to the group of cellulolytic bacteria, which are responsible for fibre digestion and utilisation in the rumen. The genetic manipulation of ruminal fibrolytic bacteria with the aim of enhancement of their fibre-digesting ability is one of the proposed mechanisms for improvement of feedstuff utilisation efficiency and animal production [2]. One of the crucial steps in recombinant DNA technology of ruminal bacteria is the establishment of a stable DNA transfer system. Since there is known data on transformation of ruminal bacteria *B. fibrisolvens* [3], recombinant butyrivibrios seem to be a promising tool for manipulation of ruminal processes.

Because of possible transformation of these bacteria with plasmid vectors, there is a need to characterise their DNA protection system. The great heterogeneity of *B. fibrisolvens* observed by comparison of 16S rDNA sequences [4] raises questions whether there is some association between bacterial diversity and restriction endonuclease variability.

In bacteria, site-specific (restriction) endonucleases are usually accompanied by a modification methyltransferase having the same specificity, forming a restriction–modification system. Those systems are believed to represent the main bacterial protection system against bacteriophage infections. Phage (or plasmid) DNA entering a bacterial cell is rapidly degraded by the restriction endonuclease, while host DNA is protected by appropriate methylation. As numerous DNA sequences could serve as targets for restriction, the specificities of restriction endonucleases

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are considerably variable. Up to now, some 270 specificity groups (see REBASE database available via http://rebase.neb.com) were described in bacteria and viruses from all taxonomic and ecological groups.

Ruminal bacteria, probably as a result of selection pressure provided by high numbers of bacteriophages in the ruminal ecosystem [5], are frequently found to possess restriction–modification systems. There is little known about restriction–modification systems of *B. fibrisolvens* strains. To date, only two restriction endonucleases, Bfi57I and Bfi89I, were described in this species [6].

The aim of present study was to analyse endonuclease variability in ruminal bacteria identified previously as *B. fibrisolvens*.

2. Material and methods

2.1. Bacterial strains and media

Pure bacterial strains were isolated from the rumen fluid and faeces of herbivorous animals under anaerobic conditions [7] and from different culture collections [8]. We greatly appreciate the gifts of G.T. Attwood (Palmerson, New Zealand), K. Gregg (Perth, WA, Australia), N.O. Van Gylswyk (Uppsala, Sweden) and C.S. Stewart (Aberdeen, UK). The bacteria were cultivated at 39 °C in M10 medium [9] containing 10% rumen fluid and 0.4% cellobiose as the carbon source. The following methods were used for the identification of bacterial strains: fermentation products were analysed on Shimadzu GC-14A gas chromatograph [10], substrate utilisation and degradation of biopolymers was estimated with API 20 and ID 32 kit (BioMerieux, France) and the cellular fatty acid composition was determined by gas chromatography [8]. Genomic DNA was isolated [11] and 500 bp long fragments of 16S rDNA were sequenced with primers FP27/515R [12] on ABI 310 capillary sequencer (Perkin-Elmer). The G + C content of bacterial chromosomal DNA was estimated by HPLC chromatography on C18 reversed-phase column (Supelcosil LC18, Supelco) [13].

2.2. Screening for presence of endonucleases

The crude cell extract was prepared from 10 ml of overnight grown cultures. Cells were collected by centrifugation at $6000 \times g$ for 10 min at 4 °C. All following operations were performed on ice. The cell pellet was resuspended in sterile sonication buffer (20 mM Tris-HCl, pH 8.0; 1 mM EDTA; 10 mM β-mercaptoethanol) and cells were disrupted by 5 cycles of 30-s sonication. The bacterial DNA was precipitated by addition of streptomycin sulphate to a final concentration 2% and removed by centrifugation ($12{,}000 \times g$, 10 min, 4 °C). The proteins in supernantant were concentrated with polyethylene glycol 6000 (SERVA, Germany) to a final concentration of 10% in phosphate buffer (20 mM Na_2HPO_4, pH 7.2) [14]. The precipitate was dissolved in 20 µl of phosphate buffer and used for screening of restriction activity on non-methylated λ DNA (New England Biolabs, Beverly, USA) as a substrate. The digestion was performed for 90 min at 39 °C in 20 µL reaction mixture of the following composition: 2 µL of protein extract, 2 µL of 10 × Invitrogen restriction buffer A (buffer contains 330 mM Tris-acetate, pH 7.9, 100 mM Mg acetate, 660 mM potassium acetate, 5 mM dithiothreitol), 1 µL of substrate and 15 µL of sterile dH_2O. The resulting fragments were separated by horizontal agarose gel electrophoresis in Tris-borate buffer [15].

All strains were screened just once except those that showed site-specific (restriction) nuclease activity. In these cases the isolation was repeated 6 months after the first experiments.

2.3. Detailed analysis of endonucleases

For detailed analysis the restriction enzymes were purified from 100 mL of overnight grown culture. The cell wall was disrupted by sonication and the cell-free extract was loaded on 5 mL HiTrap Heparin column (Amersham Biosciences, Uppsala, Sweden), which captured the endonucleases. The bound proteins were eluted with a 0–1.2 M NaCl stepwise gradient in sonication buffer (by 0.5 M increments). Ten fractions of 0.5 ml were collected and tested for the presence of endonucleases. The fractions exhibiting restriction activity were stabilised with glycerol (50%) and BSA (0.1%) and stored at −20 °C. This solution was used for enzyme characterisation by restriction of different substrates in commercially available reaction buffers A, L, M, H (Roche Applied Science, Germany). The enzymes were assayed by using a standard protocol (Roche Applied Science Product Catalogue, 1990/1991), i.e. 1 µg of DNA substrate was cleaved for 90 min at 39 °C in 10 µL total volume with 2 µL of enzyme.

The recognition sequence of purified endonucleases was identified by a series of single and double digests of several known DNA substrates (λ DNA, pSP64, pKRX and pLITMUS38 plasmids) and by comparison of the resulting restriction patterns with those theoretically generated by known restriction endonucleases (Table 2, isoschizomer column).

2.4. DNA isolation and analysis

Digestion of genomic bacterial DNA from pure strains was used for determination of modification activities. The genomic DNA was isolated from bacteria grown overnight by using the enzymatic lysis method