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ANALYSIS OF COMPLEX MICROBIAL ECOSYSTEMS WITH *IN SITU* HYBRIDIZATION AND EPIFLUORESCENT MICROSCOPY

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A B S T R A C T

In situ hybridization with fluorescent oligonucleotide probes combined with epifluorescent microscopy was used to detect and localize microorganisms in rumen samples from a black and white Friesian cow. Bacterial cells were hybridized with 16S rRNA targeted oligonucleotide probes specific for the phylogenetic group *Cytophaga-Flexibacter-Bacteroides* (CFB) (BACPRE) and for species *Prevotella bryantii* $B_1$4 ($PBB_1$4) and *P.ruminicola* 23^T (PR23). The oligonucleotide probes were labelled with fluorescine isothiocyanate (FITC) or tetramethylrodamine isothiocyanate (TRITC). Both species specific probes proved to be highly specific and gave strong and clear signal.

Key words: microbiology / bacteria / *Prevotella* / molecular biology / *In situ* hybridization / epifluorescent microscopy / rumen

ANALIZA KOMPLEKSNIH MIKROBNIH EKOSISTEMOV Z *IN SITU* HIBRIDIZACIJO IN EPIFLUORESCENTNO MIKROSKOPIJO

I Z V L E Č E K

Fluorescentno označene 16S rRNK oligonukleotidne sonde smo v kombinaciji z *in situ* hibridizacijo in epifluorescentno mikroskopijo uporabili za specifično odkrivanje bakterij v vzorcih vampne vsebine krave črno-bele pasme. S široko specifično sondo BACPRE smo odkrivali bakterijske celice iz filogenetske skupine *Cytophaga-Flexibacter-Bacteroides* (CFB*). Z* ozko specifičnima sondama pa smo specifično odkrivali bakterijske celice iz vrst *Prevotella bryantii* $B_1$4 (sonda $PBB_1$4) in *Prevotella ruminicola* 23^T (sonda PR23). Oligonukleotidne sonde smo označili s fluorescin izotiocianatom (FITC) ali s tetrametil izotiocianatom (TRITC). Sondi $PBB_1$4 in PR23 sta visoko specifični in omogočata jasen in močan signal brez spremljajoče nespecifične fluorescence.

Ključne besede: mikrobiologija / bakterije / *Prevotella* / molekularna biologija / *In situ* hibridizacija / epifluorescentna mikroskopija / vamp

INTRODUCTION

The rumen is an active and complex microbial ecosystem harbouring various important groups of microorganisms. Most of them are obligatory anaerobic and require very complex media for survival and growth (Avguštin, 1994). The detection and identification of bacterial species in the rumen ecosystem using classical microbiological methods, which require isolation of pure cultures, cultivation in vitro and characterisation of microorganisms, is difficult and time consuming (Moore and Moore, 1995). These methods can not distinguish among microorganisms on the species level. In contrast, modern molecular biology techniques enable the identification of specific microbial populations without cultivation, and directly in their environments (Amann *et al*., 1990, 1995). In situ (whole-cell) hybridization in combination with fluorescent 16S rRNA targeted oligonucleotide probes is a suitable tool for specific identification and localization of bacterial cells in their natural microhabitat environments (Amann *et al*., 1995). In situ 16S rRNA probing has been successfully used for localization of specific bacteria in a number of environments, including biofilms, plant roots and aquatic mesocosms (Jansson and Prosser, 1997).

The aim of this study was to investigate the applicability of *in situ* hybridization with fluorescently labelled 16S rRNA targeted oligonucleotide probes for specific detection of microorganisms in the rumen.

MATERIAL AND METHODS

Bacterial strain *Prevotella bryantii* $B_1$4 was grown in M2 broth at 37˚C as described previously (Tepšič and Avguštin, 1997). Cells were harvested at mid-logarithmic phase and discarded from the growth medium. The peleted cells were washed twice and then resuspended in 1ml of an ice cold PBS buffer. The rumen sample from the black and white Frisian cow was obtained and treated as previously described (Tepšič and Avguštin, 1997). Cells in the supernatant were fixed in PFA/PBS solution and then stored in 1:1 mixture of PBS/ethanol.

The following oligonucleotides were used in this study**:** (a) universal eubacterial probe EUB 338 (5'-GCTGCCTCCCGTAGGAGT-3') (Amann *et al*., 1990) (b) probe specific for phylogenetic group *Cytophaga-Flexibacter-Bacteroides* BACPRE (5'-TCACCGTTGCCGCGCTACTC-3') (c) $PBB_1$4 (5'- CGCTTCCTGTGCACTCAAGT- 3') specific for rumen bacterial species *P.bryantii* and PR23 (5'-CCAACATCGAACTCACTCAAGAT-3') specific for rumen bacterial species *P.ruminicola* 23^T (Avguštin *et al*., 1994). EUB 338 and BACPRE probes were labelled at 5'end with fluorescent dye tetramethylrhodamine isothyocianate (TRITC) (MWG Biotech, Ebersberg, Germany). PR23 was labelled at 5' end with fluorescent dye fluorescine tetramethyl isothyocianate (FITC) (MWG Biotech, Ebersberg, Germany) and $PBB_1$4 probe was labelled at 5'end either with TRITC or FITC.

Fixed cells were spotted on microscopic slides (Superfrost plus slides, MJ Research, inc.), air dried and dehydrated in 50%, 80% and 96% (v/v) ethanol. *In situ* hybridization was performed at 46-50^{o}C in hybridization buffer containing 0.9 M NaCl, 0.1% SDS, 20 mM-Tris/HCl (pH 7.4) for 3-16 hours. Probe concentrations were 5 ng/µl. The hybridization mixture was then removed by immersing the slide for 20-40 min in hybridization buffer at 48-50^{o}C, followed by thorough rinsing with sterile distilled water. The slides were air dried and viewed after being embedded in antifade mountant citifluor (Citifluor ltd., Canterbury, United Kingdom).

Fluorescence was detected with epifluorescent microscope (Olympus Optical Co., Japan) with filter set systems: U-MSWB, U-MSWG and U-MWB. Colour photomicrographs were done with Kodak Ektachrome ASA 400/27^{o}C and Fujichrome Reversal Sensia ASA 400/27^{o}C films.

Exposure times were 0.42 - 4.52 s for phase contrast and 1.29 - 14 s for epifluorescent micrographs.

RESULTS AND DISCUSION

Individual rumen gram negative bacterial cells were specifically detected and identified in rumen liquid samples with rRNA-targeted oligonucleotides end labelled with fluorescent dyes. Members of the phylum *cytophaga-flexibacter-bacteroides* were specifically detected with the broad BACPRE probe. Bacterial cells belonging to the species *P.bryantii* ($B_1$4) and *P.ruminicola* (23^T) were specifically detected with species specific probes $PBB_1$4 and PR23 respectively. Universal bacterial probe EUB 338 was used as a positive control assuring that the bacterial cells possessed undegraded ribosomes and therefore the targets in an accessible state. Cells in the rumen fluid samples collected from the cow rumen as previously described (Tepšič and Avguštin, 1997) were first separated from the plant material, fixed and hybridized. Fluorescent signal of the probes which specifically annealed to the complementary sequences on the ribosomes of the fixed cells (Wilkinson, 1992) was detected with epifluorescence microscopy.

The figure 1 shows the selective identification of $PBB_1$4 probe which specifically identified only the rumen bacterial cells belonging to the species *P.bryantii* $B_1$4 in a rumen liquid sample with added $B_1$4 laboratory culture cells. Non-specific binding of oligonucleotides was not observed when a number of nonrelated bacteria was exposed to *in situ* hybridization with $PBB_1$4 probe (not shown). The fluorescence within the cells was clearly distinct from the dark background. Surprisingly, the target cells from the rumen fluid seem to be 2-3 times smaller than the added cells from the pure laboratory culture. This phenomenon could be explained with favourable growth conditions in a laboratory culture compared with those in the rumen (excess of nutritive substances, absence of competition).

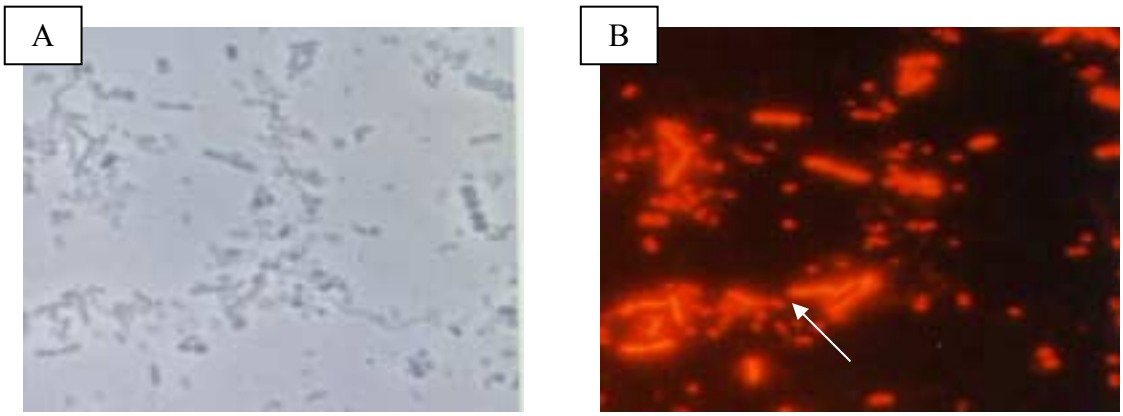

Figure 1. Rumen liquid sample with added pure culture *P.bryantii* $B_1$4 cells (arrow). A: phase-contrast microphotography. B: epifluorescent microphotography following the *in situ* hybridization with the $PBB_1$4 probe labelled with TRITC.

Slika 1. Vzorec vampnega soka z dodanimi celicami čiste bakterijske kulture *P.bryantii* $B_1$4. A: fazno kontrastna mikrofotografija. B: epifluorescentna mikrofotografija po *in situ* hibridizaciji s sondo $PBB_1$4, označeno s TRITC.

On Figure 2 the difference in size between the *P.bryantii* $B_1$4 cells grown on M2 medium and the cells grown on modified M2 medium, containing oat spelts xylan rather than soluble sugars, can be observed. The growth on soluble sugars obviously enables not only the faster growth

(Gasparič *et al*., 1995) but also the formation of larger cells than growth on a complex substrate such as xylan.

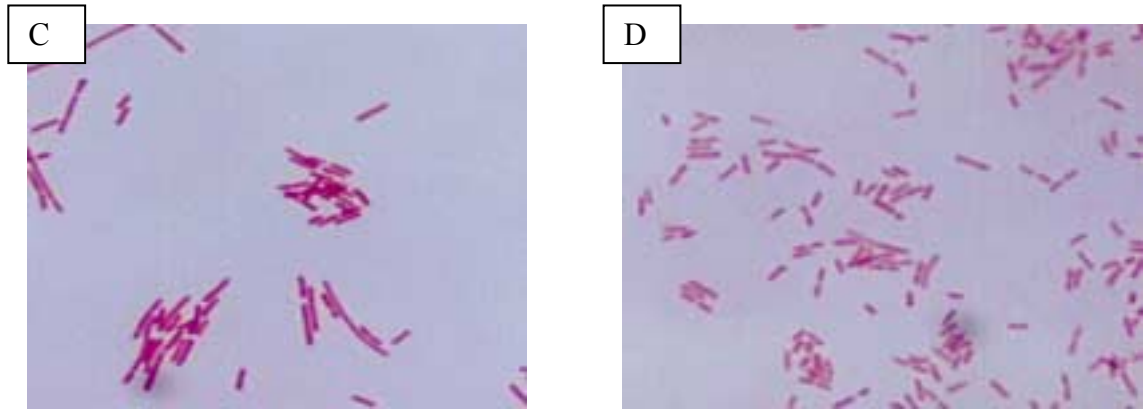

Figure 2. Gram stained cells of pure culture *P.bryantii* $B_1$4. C: *P.bryantii* $B_1$4 cells were grown on M2 medium D: *P.bryantii* $B_1$4 cells were grown on M2 medium with added oat spelts xylan.

Slika 2. Po gramu obarvane celice čiste bakterijske kulture *P.bryantii* $B_1$4. C: Celice *P.bryantii* $B_1$4 gojene na M2 rastnem gojišču. D: Celice *P.bryantii* $B_1$4 gojene n M2 rastnem gojišču z dodanim ksilanom.

When rumen liquid sample was analysed, a large part of the cells was recognized by the BACPRE probe (Tepšič and Avguštin, 1997). However, the exact enumeration of the cells proved to be almost impossible without automatic image analysis system, due to uncontrolled and nonhomogenious distribution of cells on the microscope slide during the fixation procedure. The basis of this phenomenon may well be the specific coating of the slides used for *in situ* hybridization which secures that cells remain adhered on the glass during the hybridization and washing procedures.

Therefore, the alternative enumeration method was sought, allowing equally fast and specific enumeration of bacterial cells in complex microbial ecosystems i.e. rumen. Competitive PCR (cPCR) seems to be the method of choice if the methodology of the PCR product quantification is available (Reilly and Attwood, 1998). The cPCR system for exact enumeration of *P.bryantii* $B_1$4 cells is being developed and will hopefully in combination with *in situ* hybridization and epifluorescent microscopy allow exact enumeration, monitoring and specific localisation of targeted bacterial cells in complex microbial ecosystems.

POVZETEK

Fluorescentno označene oligonukleotidne sonde rRNK v kombinaciji z *in situ* (whole-cell) hibridizacijo in epifluorescentno mikroskopijo smo uspešno uporabili za specifično odkrivanje in lociranje bakterijskih celic v kompleksnem vampnem ekosistemu. Vampne bakterijske celice so po fiksaciji in dehidraciji dostopne za oligonukleotidne sonde in vsebujejo dovolj tarčnih molekul rRNK. Uporabljeni sondi $PBB_1$4 in PR23, označeni z fluorescin izotiocianatom (FITC) oz. tetrametilrodamin izotiocianatom (TRITC), sta visoko specifični in dajeta močan in jasen signal, kar omogoča enostavno odkrivanje z epifluorescentno mikroskopijo. Bakterijske celice v vzorcu vampne vsebine so v primerjavi s celicami čiste bakterijske kulture manjše, kar lahko razložimo z različnimi rastnimi pogoji, vendar po hibridizaciji s sondo $PBB_1$4 svetijo z enako jakostjo. *In situ* hibridizacija je primerna metoda za ekološke analize kompleksnega vampnega

ekosistema, ki pa bi v kombinaciji s kvantitivnimi metodami (kvantitativna verižna reakcija s polimerazo) ter z digitalno obdelavo mikroskopske slike omogočila natančnejši vpogled v vampni ekosistem.

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