

Application of the Differential Display RT-PCR Technique to Examine Conditional Gene Expression in *Ruminococcus albus*

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ABSTRACT

In vivo expression technologies and differential display RT-PCR provide new approaches to further examine a microbe's response to experimental conditions which more closely resemble natural microbial associations and habitats. The latter technique also expands the scope of research which can be conducted with bacteria for which there is limited or no genetics protocols. We have used differential display RT-PCR to examine the physiological response of *Ruminococcus albus* to phenyl-substituted fatty acids. Micromolar concentrations of these acids have previously been shown to dramatically improve the ability of this bacterium to utilize cellulose as a carbon and energy source, thereby improving the environmental fitness of this bacterium in the ruminal environment. Coupled with northern blot analysis, we have been able to identify several transcripts which increase in abundance in response to phenyl-substituted fatty acids. Preliminary cDNA sequence analysis suggest that the transcripts encode enzymes, regulatory and transport proteins which have not been previously identified in this bacterium. It should be possible to further expand the application of differential display RT-PCR to study other aspects of physiological ecology and host-microbe interactions.

Introduction

Ecology in its broadest context is the study of the relationships of organisms with animate and inanimate characteristics of their environment (Thain and Hickman, 1995). Accordingly, one aspect of microbial ecology research is to examine the adaptive physiological responses of a microbe, or group of microbes, to perturbations in their growth environment. With the development of techniques in bacterial genetics and molecular biology, it has been possible to delineate at a very precise level the mechanisms coordinating a bacterium's response to changes in its growth environment. Our depth of understanding is generally proportional with the sophistication of the above mentioned techniques that may be utilized with a particular microbe. Even though relatively little is understood about adaptive physiological responses in those microbes for which genetics-based analyses has not been an option; it is also clear that much is still to be learned about microbes which have been the focus of intense study. The completed microbial genome sequencing projects have revealed that as many as 30% of the presumptive coding sequences in prokaryote genomes have no current match in databases and therefore, are of unidentified function (Hinton, 1997). Recent advances in molecular biology facilitate new opportunities to identify and ascribe function to these so-called "FUN" genes. Both *in vivo* expression technology (IVET) and differential display reverse transcriptase-polymerase

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chain reaction (DD RT-PCR) have permitted the identification of stress-, acid-, starvation-, and colonization-inducible genes in pathogenic bacteria (Wong and McClelland, 1994; Valdivia and Falkow, 1997). While the former is applicable with bacteria for which relatively sophisticated genetic techniques are available; DD RT-PCR can be applied to microorganisms for which little or no techniques in bacterial genetics have been developed. However, there currently appears to have been limited application of this approach other than in the study of bacterial pathogenesis.

We have begun to utilize DD RT-PCR as part of our studies of *Ruminococcus albus*, a gram-positive anaerobe best recognized for its role in cellulose degradation in ruminant animals. The cellulase system of this bacterium is distinctive relative to better characterized cellulolytic bacteria such as *Clostridia* spp.; it has long been recognized that growth of the bacterium with cellulose as the sole carbon and energy source is dependent on the provision of ruminal fluid in the growth medium. Stack, Hungate and coworkers later demonstrated that the bacterium required micromolar concentrations of phenylacetic (PAA) and phenylpropionic (PPA) acids to stimulate cellulase enzyme production and cellulose digestion kinetics. Cell morphology is also altered in response to micromolar concentrations of PAA and PPA, with both vesicular and fimbrial structures produced, and cellulases remain associated with the bacterial capsule (Stack and Hungate, 1984). We hypothesize that these phenyl-substituted fatty acids serves as a signalling molecule for *R. albus*, and co-ordinate expression of genes critical to degradation and growth on cellulose. We present here some of our initial results using DD RT-PCR to examine and identify differentially expressed transcripts in response to these compounds.

Methods

RNA isolation and DD RT-PCR procedures.

We first developed an RNA extraction protocol for use with *R. albus* that is both rapid and provides a relatively high yield of undegraded RNA. The procedures and details are described in Pegden *et al.* (1998). A key element in the protocol has been to ensure that the initial steps of cell concentration and resuspension in lysis buffer are performed under anaerobic conditions. Cell lysis is mediated by the use of mutanolysin, proteinase K and SDS. Once cell lysis is complete, RNA is recovered using the RNeasy Purification System from Qiagen according to manufacturer's recommendations. Using this procedure we routinely obtain approximately 30 μ g RNA from a 5 ml broth culture of cells harvested at mid-log phase of growth. For the experiments described here, *R. albus* was grown in EM medium (Pegden *et al.*, 1998) supplemented with either 5% (vol/vol) clarified ruminal fluid, filter sterilized solutions of both PAA and PPA to provide a final concentration of 25 μ M for each, or sterilized water.

The DD RT-PCR procedure was essentially the same as described by Liang *et al.*, (1993) except RT of the DNA-free total RNA was performed with random hexamers, and [α -³³P] dATP was used in PCR amplification of cDNA. To confirm that the RNA was not contaminated with DNA, PCR reactions were carried out with RT samples which contained no reverse transcriptase. Both the RT and PCR reactions were done in duplicate, and the cDNA products resolved on 6% (wt/vol) polyacrylamide gels under denaturing conditions. In initial reactions, PCR was performed with 20-mer oligonucleotides, specific for the *R.albus cbpC* (cellulose binding protein C) gene (Pegden *et al.*, 1998). In subsequent experiments, 10-mer oligonucleotides (Operon Technologies, Inc.) were

chosen on the basis of limited sequence homology with the 16S rDNA gene of *R. albus* strain 8.

Reamplification and northern blot analysis

Putative differentially displayed bands were excised from polyacrylamide gels, and the DNA was reamplified using the same primer and PCR conditions. The DNA was ethanol precipitated and cloned into the pCR 2.1 TA cloning vector (Invitrogen). The nucleotide sequence of the cloned DNA was obtained using universal T7 and M13 reverse primers. The cloned DNA was then used as a template to produce [³²P] dCTP-labeled probes by random-primer labeling. For northern blot analysis, 20 µg of total RNA for each growth condition was fractionated in 1% (wt/vol) agarose gels in the presence of both glyoxal and dimethylsulfoxide following standard procedures. The RNA was transferred to Zeta-Probe GT membrane by vacuum blotting and immobilized by UV-crosslinking. Blots were hybridized overnight at 43°C and washed twice at 43°C for 30 minutes in 40 mM disodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% (wt/vol) SDS. Differences in RNA loading were determined by stripping the blots and reprobing with the ³²P end-labeled 18-mer oligonucleotide (5'-TACCGCGGCTGCTGGCAC-3') that is complementary to virtually all known 16S rRNA sequences (Zheng *et al.*, 1996).

Results

RT-PCR of the cbpC gene and detection of differentially expressed transcripts

The use of the *cbpC*-gene specific primers in RT-PCR resulted in the expected amplification of a 350 bp fragment of the *R. albus cbpC* gene (Pegden *et al.*, 1998) for all DNase-treated RNA preparations. This control provided us with confidence that the DNase-treated RNA used in subsequent DD RT-PCR was of high quality. A typical autoradiograph of the DD RT-PCR reactions is shown in Fig. 1. Eighteen putative differentially expressed sequence tags (dESTs) and 2 constitutive ESTs (cESTs) were further examined by northern blot analysis. Of the 18 putative dESTs, 6 were confirmed to be differentially expressed, and the results of BLAST alignments are shown in Table 1. Three dESTs (D4, D9, and D11) hybridized to transcripts present in greatest abundance in cells grown with PAA and PPA. One dEST (D3) hybridized to a transcript that was upregulated during growth with ruminal fluid; another dEST (D15) identified a transcript present when neither rumen fluid or PAA and PPA were added to the growth medium. The last dEST (D18) identified two transcripts, both in greater abundance when either ruminal fluid or PAA and PPA were included in the growth medium. As expected, the abundance of transcripts recognized by the cESTs were similar for all growth conditions. Interestingly, transcript size for most of the dESTs were greater than 4 kilobases (kb), suggesting that these transcripts are polycistronic.

Discussion

Relationship between differentially expressed transcripts and cellulose hydrolysis by R. albus

The use of DD RT-PCR has provided us with an opportunity to expand the scope of our research, to examine in more depth the physiological response(s) of *R. albus* to environmental conditions known to affect degradation of, and growth on cellulose. We are

Table 1. Preliminary BLAST alignments of differentially and constitutively expressed transcripts.

EST	Putative Homolog	Amino Acid Identity	Probability Value
D3	glutathione peroxidase	32/57 (56%)	7.1×10^{-13}
D4	ABC exporter accessory factor	23/89 (25%)	0.0015
D9	amidophosphoribosyltransferase	108/189 (57%)	2.0×10^{-57}
D11	surface layer protein B	31/114 (27%)	3.0×10^{-04}
D15	no matches in databases	-----	-----
D18	integral membrane protein	19/43 (44%)	5.0×10^{-06}
C1	pyruvate dehydrogenase	11/23 (47%)	0.031
C2	dolichol phosphate mannose synthase	16/24 (66%)	0.022

currently focussing on two dESTs, D4 and D18. The D4-specific probe hybridizes to a transcript of approximately 6 kb, and preliminary BLAST alignments indicate the available sequence shares identity with an ABC exporter accessory protein from *Lactobacillus sake* and a hypothetical protein from *Bacillus subtilis*. The BLAST alignments using the D18 dEST identified a strong match with an integral membrane protein (*cglF*) from *Thermoanaerobacter brockii*. The *cglF* gene appears to be part of an operon which also encodes for a cellodextrin-cleaving beta-glucosidase (*cglT*) and xylodextrin-cleaving xylo-beta-glucosidase (*xglS*) (Breves *et al.*, 1997). Operons, commonly found in the genomes of both eubacteria and archaea, often contain genes associated with the same physiological function. In light of the knowledge that both PAA and PPA have been shown to enhance the secretion of cellulase enzyme activity to the bacterial cell surface and improve adhesion to cellulose, the D4 and D18 dESTs have provided encouraging clues to the identification and function of gene(s) involved in the degradation of cellulose. This knowledge will accelerate our understanding of the mechanisms involved and the role of PAA and PPA in cellulose hydrolysis by *R. albus*.

Potential limitations and caveats to the use of DD RT-PCR

Despite the positive results to date, DD RT-PCR does have several potential limitations and caveats. In addition to the success being dependent on the quality of RNA extracted from cells, there is also some bias which favors amplification of the more abundant mRNAs (Bertioli *et al.*, 1995). Accordingly, low copy number mRNAs and/or large transcripts might not be easily detected in northern blot analyses (Yeatman, 1995). Another concern is the ability to ensure complete genome coverage by the primer sets chosen for cDNA amplification. In a number of cases primer choice is being facilitated by genome sequencing projects, with the identification of oligonucleotide motifs of relatively high frequency, and their dispersion over the chromosome (Fislag *et al.*, 1997). However, to fully understand conditional gene expression and decipher function(s) encoded by such genes, other methods (such as high-density probe array systems, proteome analysis by two-dimensional protein gel electrophoresis, and reverse genetics) should allow a quantitative analysis of gene expression patterns on a genomic scale.

A genomics based approach to the dissection and characterization of regulatory pathways and networks will provide new insights into the interrelationship among genes in response to environmental signals. Although "new" genes evolved by way of duplication

and modification of "old" genes, these modifications generally occur at a slow enough pace that related genes can be determined on the basis of sequence (Doolittle, 1998). However, sequence similarity does not necessarily extrapolate to similar function and conversely,

1 2 3 4 5 6

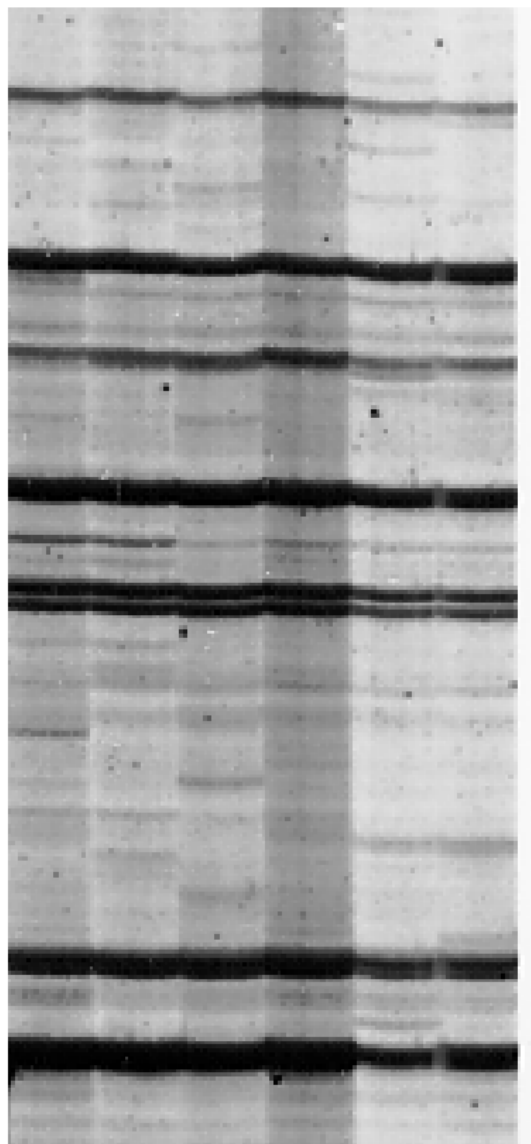


Figure 1. DD RT-PCR of RNA isolated from mid-logarithmic *R. albus* cultivated in EM-cellobiose medium with rumen fluid (lanes 1 and 2), without rumen fluid (lanes 3 and 4), and without rumen fluid, but with PAA and PPA added (lanes 5 and 6).

lack of sequence similarity does not necessarily mean unrelated function. Nonetheless, our use of DD RT-PCR has contributed valuable information about the response of *R. albus* to biochemical and physical cues, and will facilitate our efforts to develop a comparative model with other cellulolytic and(or) gram-positive bacteria.

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