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PROOF

Acholeplasma laidlawii PG8 ultramicroforms amplificate selectively *rrn*B nucleotide sequences

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Abstract - Mycoplasmas are frequent contaminants of *in vitro* animal cell cultures. Despite a broad spectrum of modern methods, detection of mycoplasmas remains a serious problem. The situation is complicated by the fact that mycoplasmas may be presented in cell cultures or biological samples by viable but unculturable forms (ultramicroforms). We found that the DNA of *Acholeplasma laidlawii* PG8 ultramicroforms showed selective amplification of the *rrn*B nucleotide sequences while vegetative cells of the mycoplasma showed amplification both for *rrn*A and *rrn*B sequences. The role of enzyme deproteinization in PCR results was also shown. The results presented in this report indicate that the optimisation of primer sequences as well as PCR regime may be crucial steps in detection and differentiation of vegetative forms and ultramicroforms of *A. laidlawii.*

Key words: polymerase chain reaction, *Acholeplasma laidlawii,* ultramicroforms, ribosomal operons.

INTRODUCTION

Mycoplasmas are frequent contaminants of *in vitro* animal cell cultures (Barile and Rottem, 1993). The usage of the mycoplasma-infected cell cultures may result in unreliable experimental results and may favour to transmission of various diseases. Therefore, it is very important to establish a reliable diagnostic protocol for mycoplasma detection. Despite a broad spectrum of modern methods (e.g., immunosorbent assays, immunofluorescence and biochemical assays), detection of mycoplasmas remains a serious problem. The situation is complicated by the fact that mycoplasmas may be presented in cell cultures or biological samples by viable but unculturable forms (ultramicroforms) (Chernov *et al*., 2005). We report here that ribosomal operons of *Acholeplasma laidlawii* PG8 (*rrn*A and *rrn*B) show a differential amplification during nanotransformation of the mycoplasma to ultramicroforms at unfavourable growth conditions. This fact may be used for detection of mycoplasma ultramicroforms that may infect cell cultures or other biological niches as mycoplasma vegetative cells do.

Acholeplasma laidlawii PG8 strain was obtained from the N.F. Gamalei Research Institute of Epidemiology and Microbiology (Moscow, Russia). *Acholeplasma laidlawii* PG8 cells were grown in the liquid-modified Edward's nutrient medium (Chernov *et al.,* 2005). Vegetative form cells and ultramicroforms of the mycoplasma culture were separated by centrifugation in density gradient of Percol (Sigma, Moscow, Russia) according to Nishino *et al.* (2003). Using the previously published data on the mycoplasma rRNA genes (Weisburg *et al.,* 1989; Kong *et al.,* 2001), we constructed A16LF (5'-GGAGGAAGGTGGGGATGACGTCAA-3'), and A23LR (5'-CCTTAGGAGATGGTCCTCCTATCTTCAAAC-3') primers complementary to gene fragments of *A. laidlawii* PG8 16S rRNAand 23S rRNA flanking spacer area of ribosomal operon. Oligonucleotides were synthesized by the scientific production company Litech (Moscow, Russia). PCR was performed as it was mentioned before (Chernov *et al.,* 2005). Analysis of homology with occasional nucleotide sequences presented in the GeneBank database showed that A16LF and A23LR primers may correspond to genus-specific probes in the interval of the calculated annealing temperatures. The sizes of the amplifying DNA fragments may be used as *A. laidlawii* species-specific markers. There were no PCR signals when DNAs extracted from some organisms (including *Pisum sativum*, *Catharanthus roseus*, *Vinca minor*, *Triticum aestivum*, *Escherichia coli*, *Bacillus subtilis*, *Clostridium perfringens*) and from samples of chernozem and podsolic soils were used as a matrix. Specifity of A16LF and A23LR primers was also confirmed in laboratory experiments.

Previously, we showed (Chernov *et al.,* 2004, 2005) that populations of *A. laidlawii* PG8 cells grown in the full Edward's medium and deficient medium differ in the proportion of cells of vegetative forms and ultramicroforms. The cell culture grown in deficient medium was represented mostly by ultramicroforms. In this study we observed the evident disproportion of PCR signals for amplicons of the nucleotide sequences of *rrn*A and *rrn*B operons of *A. laidlawii* PG8 cells grown in the deficient medium if the

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mycoplasma DNA without special enzyme deproteinization was used as a matrix in PCR. At the same time, an equal proportion of the PCR products concerning nucleotide sequences of *rrn*A and *rrn*B operons was detected at DNA amplificating the mycoplasma cells grown in the full Edward's medium. Our data suggested that the electrophoretic profile for the mycoplasma cells of vegetative forms agreed with the theoretically expected one – presence of amplicons both of *rrn*A and *rrn*B *A. laidlawii* PG8 operons characteristic of "equal" amplification. At the same time, a selective amplification of nucleotide sequences of *rrn*B operon was characteristic of the DNA of ultramicroforms. The nucleotide sequences of *rrn*A operon containing genes for tRNA of the mycoplasma did not amplify if the DNA of the mycoplasma ultramicroforms was used without enzyme deproteinization. However, when the DNA matrix treated with proteinase K was used, the amplification of the *rrn*A and *rrn*B sequences "grew up" and the electrophoretic profile of ultramicroforms corresponded to cells of vegetative forms of the mycoplasma.

We have reported (Chernov *et al.,* 2004, 2005) that transformation of *A. laidlawii* PG8 vegetative cells into ultramicroforms was accompanied by significant reorganization of the mycoplasma genome expression and other biochemical changes. The cancellation of amplification of some DNA sequences might be connected with the synthesis of special stress proteins with high affinity to DNA (Kenri *et al.,* 1998; Warner and Oliver, 1998). We consider that the reason for the local changes of the matrix properties of DNA might be mediated by alterations in its topology – supertorsion and synthesis of the DNA-binding stress proteins that might complicate primer annealing and completing of polymerase. The complex formed by the proteins with DNA being sustained at phenol extraction was sensitive to the proteinase K treatment. Really, the amplification of *rrn*A and *rrn*B nucleotide sequences of ultramicroforms "grew up" when the proteinase K-treated DNA of mycoplasma was used as a matrix in PCR with A16LF and A23LR primers: the amplification was characteristic of the vegetative cell forms of the mycoplasma. We suggest that the differential inhibition of amplification of the *rrn*A nucleotide sequences in ultramicroforms might be connected with the presence of thermoresistant DNA-binding polypeptides in the samples. The use of specific nucleotide sequences of the *rrn*A spacer area as primers for PCR, as well as the mycoplasma cell DNA without special enzyme deproteinization as a matrix, may lead to wrong conclusions about the presence of *A. laidlawii* in the tested samples. Thus, the results presented in this report indicate that the optimisation of primer sequences as well as PCR regime may be a crucial step in the reliability and sensitivity of the technique for detection of *A. laidlawii* cells able to exist as vegetative forms and/or as ultramicroforms. The presented regime for the directional amplification of the nucleotide sequences of *A. laidlawii* rRNA operons with A16LF and A23LR primers seems an efficient tool to detect and differentiate vegetative cell forms and ultramicroforms of the mycoplasma in various samples.

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