DIFFERENTIAL AMPLIFICATION OF *ACHOLEPLASMA LAIDLAWII* PG8 *rrn*A AND *rrn*B NUCLEOTIDE SEQUENCES DURING DISSOCIATION OF THE CELL CULTURE POPULATION

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Accepted for Publication November 2, 2006

ABSTRACT

The amplification of the 16S–23S spacer areas of the Acholeplasma laidlawii *PG8 rRNA* rrn*A and* rrn*B operons has its own features. The attenuation of the polymerase chain reaction (PCR) signal of the nucleotide sequences of* rrn*A containing tRNA genes might be observed if DNA without enzyme deproteinization is used as a matrix. The phenomenon takes place due to dissociation of cell population caused by the active entering of the vegetative cell forms into ultramicroforms in the mycoplasma culture at unfavorable growth conditions. The DNA of* A. laidlawii *PG8 ultramicroforms showed selective amplification of the* rrn*B nucleotide sequences – tRNA-free rRNA operon. As to vegetative cells, the "equal" PCR signals for the nucleotide of* rrn*A and* rrn*B sequences were registered. In this connection, 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. The ability of* A. laidlawii *PG8 vegetative cell forms of actively entering into ultramicroforms at unfavorable growth conditions seems to demand a new approach to control mycoplasma infections, providing an efficient diagnosis to detect the vegetative cell forms and ultramicroforms of the mycoplasma in the tested samples.*

INTRODUCTION

Mycoplasmas are the smallest and the simplest self-replicating prokaryotic organisms. The limited biosynthetic possibilities determine the depen-

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Journal of Rapid Methods & Automation in Microbiology **14** (2006) 369–376.

dence of mycoplasmas on higher organisms. During evolution, mycoplasmas adapted to the closest coexistence with cells of higher eukaryotes. In nature, all mycoplasmas are facultative parasites or commensals of plants and animals. Many mycoplasmas are pathogens of plants, animals and humans. These microbes are the main contaminants of cell cultures including those used in biotechnology to produce viral vaccines. Moreover, the same mycoplasma species may be widely spread in various biocenosis. For example, a "ubiquitous" mycoplasma, *Acholeplasma laidlawii*, is found in soil and compost, as well as in tissues of human beings, animals, insects and plants. This mycoplasma is one of the dominant contaminants of cell cultures (Razin and Herrmann 2002).

The diagnosis of mycoplasma infections and contaminations is a problem. The traditional ways to detect these bacteria (a microbiological method involving seeding on cell-free nutrient medium, and an antibody-mediated method based on detection of antibodies) are unreliable because of certain biological properties of mycoplasmas. The ideal method to detect microorganisms must be highly specific, simple and rapid. Also, it must allow the evaluation of multiple infections. In this connection, the major hopes for express testing of mycoplasmas are connected with the methods for direct amplification, e.g., polymerase chain reaction (PCR).

PCR allowing the direct amplification of nucleotide sequences of rRNA operons is now widely used to detect mycoplasmas including *A. laidlawii* in various samples (soil isolates, cell cultures, tissues of plants, animals and humans). A combination of conservative and variable regions of 16S and 23S spacers of rRNA operons determines their wide use as genus- and species-specific markers and diagnostic probes of microorganisms. In *A. laidlawii* genome, there are two rRNA operons with genes for rRNA 23S, 16S and 5S subunits – *rrn*A and *rrn*B (Weisburg *et al.* 1989; Nakagawa *et al.* 1992; Kong *et al.* 2001). Two tRNA genes – for isoleucine and alanine – are situated in the 16S–23S spacer of *rrn*A operon. Previously, we showed (Chernov *et al.* 2005) that at unfavorable conditions of growth (substrate deficiency), cell populations of the mycoplasma dissociated due to actively entering vegetative cell forms into ultramicroforms. Meanwhile, in regard to some amplifying regions of bacterial genome experiencing the unfavorable conditions of growth (including the substrate limitation), a reversible effect of the PCR signal attenuation was observed (Warner and Oliver 1998). Primers for the direct amplification of one or another nucleotide sequences of *rrn*A and *rrn*B 16S–23S spacers are used for the detection of *A. laidlawii* (Kong *et al.* 2001). The present study was intended to test PCR signals at amplificating 16S–23S spacers nucleotide sequences containing *rrn*A and *rrn*B operons of *A. laidlawii* PG8 when cultivating at unfavorable growth conditions (nutrient substrate deficiency).

MATERIALS AND METHODS

A. laidlawii PG8 strain was obtained from the N.F. Gamalei Research Institute of Epidemiology and Microbiology (Moscow, Russia). *A. laidlawii* PG8 cells were grown in the liquid-modified Edward's nutrient medium (Chernov *et al.* 2005). Tryptic heart extract, serum of horse blood (All-Russia Scientific Research Veterinary Institute, Kazan, Russia), glucose (Dalchimpharm, Khabarovsk, Russia) and penicillin (Synthesis, Kurgan, Russia) were used to produce the growth medium. To induce unfavorable growth, the substrate was limited. Glucose and yeast extract were eliminated from the Edward's medium; the mycoplasma cells were cultivated on the impoverished medium from 7 days to 2 years (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 rRNA and 23S rRNA flanking spacer area of ribosomal operon (Fig. 1). Oligonucleotides were synthesized by the scientific production company Litech (Moscow, Russia).

PCR was performed in a reaction mix consisting of fivefold buffer (335-mM Tris-HCl, pH 8.8; 83-mM $(NH_4)_2SO_4$; 12.5-mM $MgCl_2$; 10.5% Tween 20; 0.5 μg/mL of gelatin), mix of deoxyribonucleoside triphosphate, 1 μL of DNA matrix (from 0.01 to 200 millimicrog) and 0.4 μM of each primer. Taq-polymerase (Litech) was added to the reaction mix before beginning the reaction in concentrations recommended by the producer. The final volume of the reaction mix was 30 μL. Fifty to one hundred microliters of mineral oil (Sigma) was thickened on reaction mix. DNA denaturation was performed at 95C for 5 s, primer annealing – at 61C (5 s), synthesis of new DNA chain – at 72C for 20 s. The reaction regimen (28–32 cycles) was controlled with the use of "Tercyc" amplifier (DNA-Technology, Moscow, Russia). In the first cycle, the time of denaturation was increased by 30 s. In the final polymerization cycle (at 72C), the time was increased up to 60 s followed by storage at 10C.

Electrophoretic separation of DNA fragments stained with ethidium bromide was performed in 1–2% agarose gel (Dia-M, Moscow, Russia) and analyzed using gel documentation (Dia-M).

RESULTS

The results of PCR experiments using DNA of the mycoplasma cells grown in the full Edward's medium and deficient medium are presented in

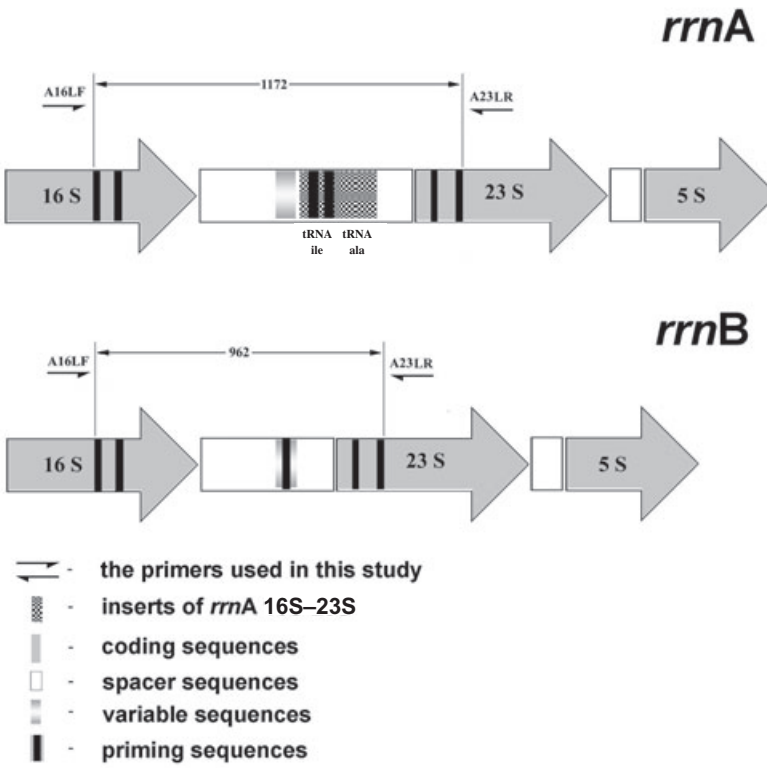

FIG. 1. THE SCHEME OF STRUCTURAL ORGANIZATION OF *ACHOLEPLASMA LAIDLAWII* *rrn*A AND *rrn*B AND AREAS FOR AMPLIFICATION OF THE NUCLEOTION SEQUENCES WITH PRIMERS A16LF AND A23LR

Fig. 2. The evident differential amplification, a 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, was observed if the 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.

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. The cells of vegetative form and ultramicroforms differed in morpho-

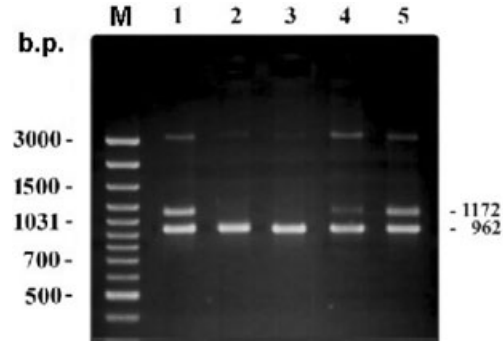

FIG. 2. DIFFERENTIAL AMPLIFICATION OF *rrn*A AND *rrn*B NUCLEOTIDE SEQUENCES OF *ACHOLEPLASMA LAIDLAWII* PG8 CELLS

Lanes 1–5 contain polymerase chain reaction of DNA extracted from the mycoplasma cells grown in full Edward's medium (1) and grown at unfavorable conditions (2,3 – unproliferated culture after 90 and 480 days of starvation, 4 – reverting culture – first passage after starvation during 480 days, 5 – active proliferating culture – first passage after starvation during 480 days).

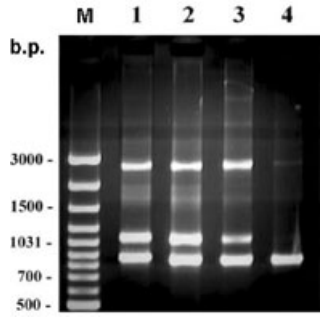

FIG. 3. REVERSIVE ATTENUATION OF AMPLIFICATION OF *rrn*A NUCLEOTIDE SEQUENCES OF *ACHOLEPLASMA LAIDLAWII* PG8 CELLS

Lanes 1–4 contain products of polymerase chain reaction (with primers A16LF and A23LR) of DNA extracted from vegetative cell forms of the mycoplasma (2, 3) and from ultramicroforms of reverting culture – first passage (after starvation during 480 days) on Edward's medium (1, 4) with (1, 2) and without (3, 4) enzymatic deproteination.

logic, ultracytostructural and molecular genetic properties. The mycoplasma cells of the two subpopulations were extracted and separated by centrifugation in density gradient of Percol. Figure 3 presents the PCR results of DNA samples of the "light" cell fraction of reversing culture (the first passage in the full Edward's medium after a 480-day cultivation in deficient medium) corresponding to *A. laidlawii* PG8 cell ultramicroforms, and "heavy" fraction

adequate for cells of vegetative forms of the mycoplasma. 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 (Fig. 3).

DISCUSSION

To compare amplification of the nucleotide sequences of *A. laidlawii* rRNA operons (*rrn*A and *rrn*B) in PCR of the mycoplasma cells during their cultivation in the full Edward's medium and deficient medium (unfavorable conditions of growth), we synthesized special primers. These primers allowed us to amplify total nucleotide sequences of the *rrn*A and *rrn*B spacer areas as well as the adjoining areas for coding regions of 16S and 23S genes (Fig. 1). A differentiated amplification of the nucleotide sequences of *rrn*A and *rrn*B of *A. laidlawii* PG8 cells grown at different growth conditions was found. Selective amplification of the nucleotide sequences of *rrn*B was marked when the DNA of ultramicroforms without special enzyme deproteinization was used in PCR (Fig. 2).

The cells of *A. laidlawii* PG8 culture grown at unfavorable growth conditions – ultramicroforms with size less than 0.2 μm – had morphologic, ultracytostructural and molecular genetic features differentiating them from the typical (vegetative form) cells of *A. laidlawii* PG8 (Chernov *et al.* 2004, 2005). On the agar nutrient medium, ultramicroforms formed specific microcolonies of less than 50–300 μm instead of the typical mycoplasma colonies ("fried eggs"). Ultramicroforms showed increased vitality and resistance to stress. Also, they were able to revert to native vegetative forms of *A. laidlawii* PG8 during subcultivation in the full Edward's medium. Entering the vegetative form of *A. laidlawii* PG8 cells into ultramicroforms was mediated by significant reorganization of the mycoplasma genome expression (Chernov *et al.* 2005).

A phenomenon of reverse attenuation of PCR signals for some bacterial cells when cultivated at unfavorable growth conditions was shown. 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). 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 (Fig. 3). This fact allows the suggestion 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.

Thus, the amplification of the 16S–23S spacer areas of the *A. laidlawii* PG8 rRNA *rrn*A and *rrn*B operons has its own features. The attenuation of the PCR signal of nucleotide sequences of *rrn*A containing tRNA genes might be observed if DNA without enzyme deproteinization was used as a matrix. The phenomenon was observed due to dissociation of cell population caused by actively entering vegetative cell forms into ultramicroforms in the mycoplasma culture at unfavorable growth conditions. The DNA of *A. laidlawii* PG8 ultramicroforms showed selective amplification of the *rrn*B nucleotide sequences – tRNA-free rRNA operon. As to vegetative cells, the "equal" PCR signals for the nucleotide of sequences of *rrn*A and *rrn*B were registered. In this connection, 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. The ability of *A. laidlawii* PG8 vegetative cell forms of actively entering into ultramicroforms at unfavorable growth conditions seems to demand a new approach to control mycoplasma infections, providing an efficient diagnosis to detect vegetative cell forms and ultramicroforms of the mycoplasma in the tested samples.

As a result of the analysis of homology with occasional nucleotide sequences presented in the GeneBank database, we found 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. So, 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 vegetative cell forms and ultramicroforms of the mycoplasma in various samples.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (Grant No. 05-04-49435), by Leading Scientific School (Head – academician I.A. Tarchevski), by the Program of Fundamental Research "Molecular and Cellular Biology" provided by the Russian Academy of Sciences and by Government Contract No. 02.442.11.7283.

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