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EXPERIMENTAL ARTICLES

Development of DNA Macroarrays for Genome Scanning of *Ureaplasma parvum* Strains

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Abstract—DNA macroarrays were developed on the basis of the known *Ureaplasma parvum* genome, which enabled rapid acquisition of the information on the changes in the microbial genome. For amplification of the PCR gene copies, 613 pairs of oligonucleotide primers were developed. Optimal conditions were determined for immobilization of the PCR products on a Nylon membrane and for hybridization with *U. parvum* chromosomal DNA. The DNA macroarrays were used to compare the nucleotide sequences of the genomes of laboratory strains of *U. parvum* and *U. urealyticum.*

Key words: Ureaplasma parvum, DNA macroarrays, hybridization, genome.

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Mollicutes (mycoplasmas) are a unique class of microorganisms characterized by a high degree of reduction of genetic material, which retained the capability of independent division on acellular nutrient media [1–4]. The absence of the cell wall (which is widespread among other bacteria), high dependence on the metabolic components obtained from the growth media, as well as high metabolic plasticity caused by the absence of a number of genomic regulatory elements, makes mycoplasmas ideal parasites of eukaryotic cells.

Members of the genus *Ureaplasma* are the only mollicutes synthesizing urease, the enzyme hydrolyzing urea to ammonia, and are unique among the *Mycoplasmataceae* in their ability to metabolize urea as an energy source. Ureaplasmas are widespread microorganisms, which colonize the mucous membranes of the urogenital tract; they are often the cause of nongonococcal urethritis [5–8], pregnancy complications, and intrauterine infections [9–11]. They may also cause infections of the central nervous system and lower airways in newborn infants [12–14]. However, ureaplasmas may be detected also in clinically healthy individuals; their role in the development of diseases is, therefore, still unclear [15].

Mucous membranes of the human urogenital tract are colonized by two ureaplasma species, *Ureaplasma parvum* (serotypes 1, 3, 6, and 14) and *Ureaplasma urealyticum* (serotypes 2, 4, 5, and 7–13) [16]. The genus *Ureaplasma* is subdivided into two species according to the following criteria: different genome size of *U. parvum* (751 kb) and *U. urealyticum* (850–1140 kb) [6, 17]; results of DNA–DNA hybridization [18]; and a high homology of the DNA nucleotide sequences within the species. Approaches used for determination of the species and serological position of the ureaplasmas include sequencing of 16S rRNA genes, the 16S–23S rRNA intragenic region, *mba* (Multiple banded antigen) of the gene, and of the A, B, and C subunits of the urease gene [19]. Attempts to establish relations between the ureaplasma serotypes and the clinical manifestations of diseases have not been successful [20–23]. We believe that the difficulty of determination of this relation may result from the macroscale variability of the ureaplasma genome (gene excision or chromosomal rearrangement).

DNA array-based whole-genome scanning [24–27] is the powerful method for obtaining primary information of the genome heterogeneity without sequencing. This method enables analyzing of numerous genes in a single experiment. Essentially, it involves immobilization of the genes of a known genome on a solid substrate and hybridization with labeled DNA of the strain under investigation is used as a probe.

The goal of the present work was development of the DNA macroarrays for genome scanning of the clinical strains of ureaplasmas.

MATERIALS AND METHODS

Cultivation of microorganisms. Laboratory strains of *U. parvum* serotype 1 and *U. urealyticum* serotype 5 were kindly provided by Prof. I.V. Rakovskaya (Gamaleya Research Institute for Epidemiology and Microbiology, Russian Academy of Medical Sciences, Moscow, Russia).

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The strains were grown for 24–48 h at 37°C in the following medium: Brain Heart Infusion (Difco, United States), 29.2 g/l; bacto trypsin (Difco, United States), 3.2 g/l; yeast extract (Medgamal, Russia), 1 vol %; horse serum (Biolot, St. Petersburg, Russia), 10 vol %; NaCl (Sigma, United States), 1.3 g/l; L-cysteine HCL, 1 vol %; amphotericin B, 1 μg/ml; phenol red, 0.5%; and pH 6.2. Microbial growth was monitored by the changing indicator color.

Primer design. Oligonucleotide primers for amplification of 613 open reading frames (ORC) of *U. parvum* was carried out with the ArrayDesigner II software package (Premier Biosoft International, United States) on the basis of the *U. parvum* serotype 3 genomic nucleotide sequence (751719 bp, GenBank accession no. AF222894) [28]. ArrayDesigner II software can work with the ORF list of the genome in the FASTA format and rate the primers according to the specified conditions (maximal level of cross and self-hybridization, optimal melting temperature, and the highest possible distance between the primers). All of the *U. parvum* ORF were also checked for possible cross homology with the BLAST software package (http://www.ncbi.nlm.nih.gov/balst/) in order to exclude the regions with 100% homology for over 50 bp in the analyzed ORF. The expected result was 70% of the 100–100 bp amplicons, 24% of 2–3 kbp ones, and 6% of amplicons of over 3000 bp.

PCR. Amplification was carried out in 100 μl of the reaction mixture containing the following: 10 mM Tris–HCL, pH 8.8; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1% Triton X-100; 1 U *Taq* polymerase; 0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP); 10 pmol of each primer; and 0.8 ng of genomic DNA of the *U. parvum* serotype 1 laboratory strain using a PTC-225 amplifier (MJ Research, United States). The amplification program was as follows: 94°C, 3 min; 94°C, 1 min; 55°C, 1 min; 72°C, 2 min (or 5 min for the fragments over 3000 bp); and the number of cycles was 35. Analysis of the PCR products was carried out by electrophoresis in 2% agarose in the presence of ethidium bromide. After amplification at primer annealing temperatures of 55–60°C, 514 PCR products were obtained. For synthesis of the remaining fragments, the primer annealing temperature was decreased to 45°C. This resulted in obtaining 68 more PCR products. Thus, the representativeness of the *U. parvum* PCR library was 95%.

Construction of *U. parvum* DNA macroarrays. Unpurified PCR products were printed to Hybond N+ Nylon membranes (Amersham Biosciences, United Kingdom) with a Qpix 2 automatic replicator (Genetix, United Kingdom) with 384 whole blunt pins 0.7 mm in diameter arranged in a format corresponding to a 384-well plate. In each experiment, the samples were applied in duplicate of ten touches each. During the printing, 40–50% humidity was maintained in the chamber. After printing the PCR products, the membranes were air-dried. For DNA fixation, the membranes were baked at 80°C for 2 h. Genomic DNA of *U. parvum* serotype 1 in six dilutions (250, 125, 62.5, 31.25, 15.6, and 7.8 pg/l) was applied to the membrane, as a control. The end product was a 7 × 12 cm Nylon membrane with 582 PCR products immobilized in a certain order in two repeats.

DNA labeling and hybridization. Chromosomal DNA of laboratory strains *U. parvum* serotype 1 and *U. urealyticum* serotype 5 isolated by phenol–chloroform extraction [29] was used for hybridization. Chromosomal DNA (25 ng) was labeled with [α-^{33}P]dATP (300 Ci/mol) using the DECA-prime II kit (Ambion, United States) according to the manufacturer's protocol.

The DNA microarray was prehybridized for 1 h in the 1× hybridization buffer (5× SSC, pH 7.5; 5× Denhardt solution; and 0.5% SDS) in Hybridization Oven/Shaker (Amersham, United Kingdom). The probe with a specific activity of 5.5×10^6 pulse min^{-1} ml^{-1} was then hybridized with the DNA microarray in 5 ml of fresh hybridization buffer for 20 h at 50, 55, or 60°C. After hybridization, DNA macroarrays were washed in 2× SSC, 0.1% SDS for 20 min, then in 0.2× SSC, 0.1% SDS for 1 h.

Coefficient of variation (CV) of the pixel intensity, i.e., the ratio of the standard deviation to the mean intensity, was used to determine whether the printing procedure and hybridization conditions were optimal [30].

DNA macroarrays were exposed for 48 h with an intensifying screen (Molecular Dynamics, United States), and scanned in a Molecular Dynamics Storm 820 unit at a 50-μmol resolution. The results were analyzed with the ImageQuant 5.1 program (Molecular Dynamics). The background was obtained as a median of signal intensities measured around spots. The signal intensity of individual spots was determined within a 0.9-mm circle, and then normalized for the summary intensity of all the points. The presence of a gene in the genome was considered proven if the corresponding signal intensity exceeded the local background value more than twofold.

RESULTS

Successful application of DNA macroarrays is determined by the optimal choice of conditions for their printing. Initially, therefore, experiments were carried out to select the solutions for immobilization of the PCR products on the Nylon membrane, since this factor determines the size and shape of the spots, subsequently manifested in a different character of the signal intensity distribution along the spot area. Several buffers for DNA immobilization on dry and pre-wet membranes were tested. After hybridization, a decrease in the total signal intensity was observed on the DNA macroarrays when NaOH-containing buffers were used for printing to both dry and pre-wet membranes. The total hybridization signal was also found to decrease

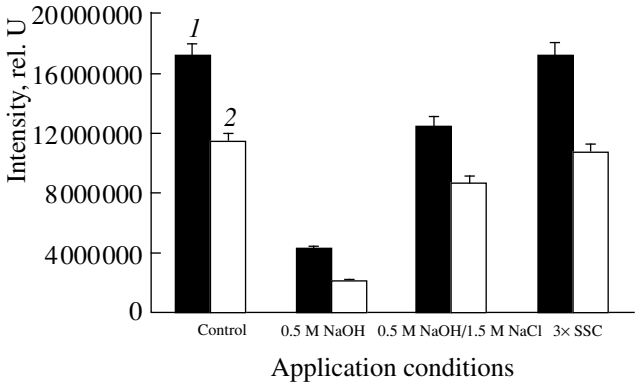

Fig. 1. Choice of solutions for immobilization of the PCR products on a Nylon membrane (*1*, moist; *2*, dry).

when the samples were printed to pre-wet membranes, rather than to dry ones (Fig. 1). No significant difference was found between the PCR products dissolved in 3× SSC and in water. For construction of the whole-genome *U.parvum* DNA macroarrays, PCR products were dissolved in 3× SSC and printed on dry Nylon membranes.

To determine the variability of the hybridization signal within a spot, intensity profiles were determined for the signal of an individual sample on dry and pre-wet filters. Arraying to a dry membrane was found to result in a sharp increase in intensity from the edge to the center of the spot, where the intensity was highest (Fig. 2a). Application to a pre-wet membrane caused uniform signal distribution along the spot area (Fig. 2b); however, as was mentioned above, this procedure resulted in a 3 times weaker signal than in the case of a dry membrane.

Hybridization temperature is another critical parameter affecting hybridization. In the case of mycoplasmas, the difficulty in DNA–DNA hybridization results from the fact that while these organisms are A+T rich, which implies low hybridization temperature (<65°C), this does not ensure specificity. Hybridization conditions were therefore tested at three temperatures, 50, 55, and 60°C. The optimal hybridization temperature was determined from the lowest values of the coefficient of variation of signal intensity between parallel hybridizations. The CV value was calculated as the ratio of the standard deviation to the mean pixel intensity. The data presented on Fig. 3 demonstrate that the lowest signal variability between the replicas occurred at 55°C.

DNA macroarrays were tested with hybridization with genomic DNA of the laboratory strain *U. parvum* serotype 1. All PCR products were successfully identified. Fig. 4 demonstrates that hybridization spots had different signal intensities. The dependence was revealed between the intensity of the hybridization signal and the length of the immobilized genome's fragments; this is probably the result of the differences in the content of labeled nucleotides in the hybridized DNA fragments. However, direct dependence between the signal intensity and the target content on the membrane was also revealed; it was confirmed in the control experiment with sequential twofold dilutions of *U. parvum* genomic DNA.

The DNA macroarrays were used for the genome scanning of laboratory strain *U. urealyticum* serotype 1. The absence of a gene was considered confirmed when the signal intensity on the DNA microarray differed from the background value by less than 2 SD (standard deviation). As a result, 380 ORF were found to be common for strains *U. parvum* serotype 1 and *U. urealyticum* serotype 5, while 175 were unique to *U. parvum* serotype 1 (table).

DISCUSSION

The genomic nucleotide sequences of microbial strains under investigation are often different from those of the strain represented in the databases. These differences may involve either single nucleotides (genomic microheterogeneity) or extensive genomic fragments including groups of polymorphic genes (macroheterogeneity), which may be responsible for

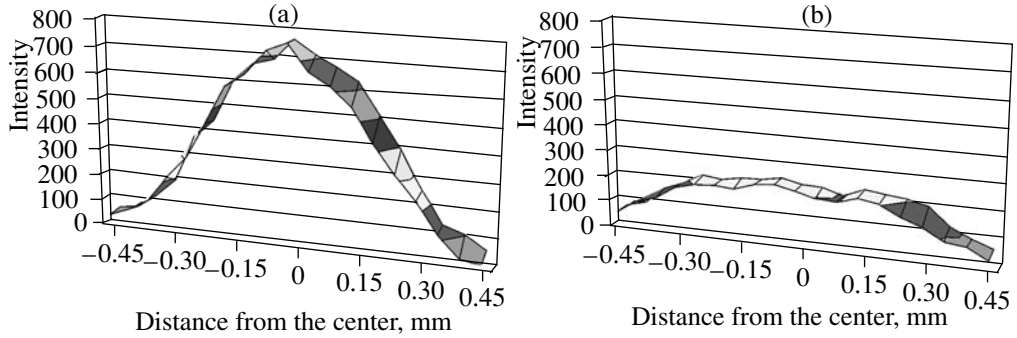

Fig. 2. Distribution profiles of the signal intensity over the sample spot of the PCR product applied to moist (a) and dry (b) membranes.

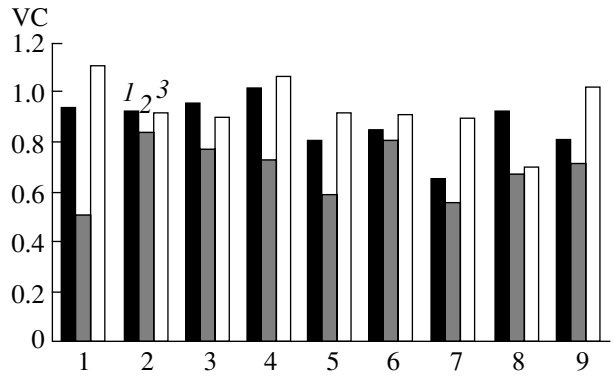

Fig. 3. Variation coefficient (VC) for the signal intensity of nine random samples at different hybridization temperatures: 50 (*1*), 55 (*2*), and 60°C (*3*).

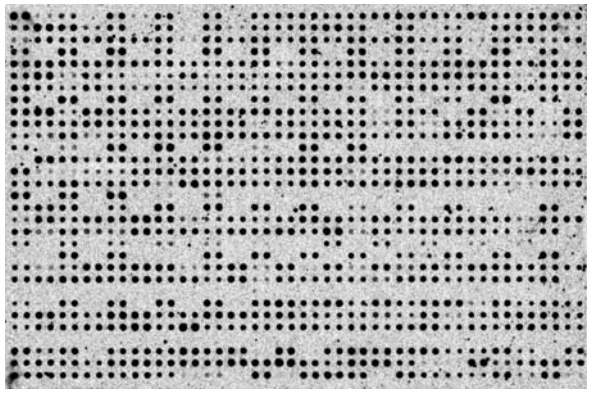

Fig. 4. Hybridization of randomly labeled ([α-^{33}P]dATP) chromosomal DNA of the *U. parvum* laboratory strain with the DNA microarray (7 × 12 cm). On the membrane, 582 PCR copies of *U. parvum* ORF are immobilized in two repeats.

the species' virulence and serve as markers for the phylogenetic and evolutionary research. These differences create some difficulties for development of whole-genome DNA macroarrays due to more complicated selection of gene-specific primers. The PCR products from all the genomic ORF are therefore impossible to synthesize. Thus, application of DNA macroarrays implies a probability of some polymorphic genes left unrecorded. However, the number of nonamplified ORF is several dozen; existence of already synthesized gene-specific primers enables determination of the presence of a gene in the genome of the investigated strain by PCR. Although PCR copies of 95% of *U. parvum* genomic ORF were obtained in the present work, we believe this number sufficient for investigation of genomic heterogeneity of different ureaplasma strains.

The choice of the solution for application is important for immobilization of the PCR products on a substrate. The ideal solution should denature the sample and minimize its evaporation, thus maintaining its concentration. Since 3× SSC does not decompose PCR products during storage and enables construction of DNA macroarrays with more densely located samples, this is the most suitable solution for application. Its main drawback is the necessity for sample denaturation after their printing to the macroarray. Printing of the PCR products to dry membranes is preferable, since the resulting DNA macroarrays have a higher sample concentration on the membrane surface, as can be seen from the signal intensity profile (Fig. 2a). Under these conditions, the signal intensity is less dependent on the sample content in the solution; preliminary concentration of the sample is therefore not required.

The *U. parvum* genome has a low G+C base content (25%), and therefore requires careful selection of the hybridization temperature. The lowest CV of the signal intensity was observed at 55°C, demonstrating its homogeneity over the integration area; high values of the variation coefficient at 50 and 60°C result from the signal heterogeneity (Fig. 3).

Hybridization on Nylon membranes with a radioactively labeled probe is the most sensitive method for operating with unpurified PCR products [31]. In the case of Nylon membranes, the signal intensity is known to depend on the amount and length of the immobilized PCR product, efficiency of the probe labeling, and hybridization conditions. The main advantage of Nylon membranes is that every sample molecule on the membrane has an equal probability of interaction with the probe; in the case of excessive immobilized PCR product on the membrane surface, this enables binding of the maximal amount of the probe. Thus, the signal intensity is proportional to the number of target molecules on the membrane and to the number of the molecules of specific probes in the hybridization solution. In the present work, random hexanucleotides were used to label the genomic DNA. The probability of the complementary binding of random primers with longer ORF may be expected to be higher, as well as the content of specific probes in the solution, resulting in higher intensity. For short ORF, the situation will be reversed. Signals of different intensity are therefore present in the hybridization picture.

The DNA macroarrays obtained and the hybridization protocol developed may be used for comparison of the genomes of different *U. parvum* strains. It should be mentioned that 175 genes unique to *U. parvum* were identified in the present work. This set of genes can be used for differentiation between *U. parvum* clinical isolates. Interestingly, 407 of 613 *U. parvum* genes were revealed in the *U. urealyticum* genome, i.e., 66% of *U. parvum* functional nucleus (407/613).

Genomic scanning of *U. urealyticum* and *U. parvum* laboratory strains

Functional group of genes	[1]Total	[2]*U. urealyticum*	[3]*U. parvum*	[4]Common
Amino acid transport and metabolism	20		5	15
Carbohydrate transport and metabolism	15		5	10
Cell cycle control	4		2	2
Biogenesis of the membrane	6		1	5
Coenzyme transport and metabolism	9		3	6
Protective mechanisms	16		5	3
Energy metabolism	16		5	11
Unknown function	28		5	21
Predicted function	45		14	31
Inorganic ions transport and metabolism	28		11	16
Intracellular transport	7		2	5
Lipid transport and metabolism	8		3	4
Nucleotide transport and metabolism	21		7	14
Posttranslational modification	19		8	11
Replication, recombination	51	1	14	33
Signal transduction	3		2	1
Transcription	19		4	14
Translation	102		16	84
No orthologes	196	26	63	94
Total	613	27	175	380

[1] Genes annotated in *U. parvum* serotype 3 (AF222894).
[2] Genes revealed only in *U. urealyticum* serotype 5.
[3] Genes revealed only in *U. parvum* serotype 1.
[4] Genes revealed in both *U. urealyticum* and *U. parvum* laboratory strains.

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