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Karyotyping of *Amoeba proteus*

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Abstract—In this paper, we have developed a method to prepare spreads of mitotic chromosomes of *Amoeba proteus* and described the process of *Amoeba proteus* karyotyping. This protocol allows obtaining spread chromosomes with a characteristic pattern of chromomeres on individual chromosomes. It is shown that, in the metaphase of mitosis, amoebas of strain B (one of the type strains of *A. proteus* in the Amoebae Cultures Collection of the Institute of Cytology, Russian Academy of Sciences) contain 27 pairs of chromosomes. It is established that the pattern of chromomeres is a chromosome-specific feature. A typical karyogram and image bank of DAPI- and YoYo1-banded individual chromosomes of *A. proteus,* strain B composed of five different spreads of mitotic cells are presented.

Keywords: Amoeba proteus, mitosis, chromosomes, karyotyping

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INTRODUCTION

Although amoebas of the species *Amoeba proteus* have been under study for two centuries, a variety of key issues in the biology of these unicellular organisms remain unexplained. Thus, all the numerous earlier attempts to determine the chromosome number of these obligate agamic protozoa do not seem satisfactory. It is still considered that the number of chromosomes in *A. proteus* reaches 500 or more (Ord, 1973; Yudin, 1990; Makhlin, 1993; Marakhova et al., 1993), but these data are only hypothetical. This is because scientists studying the karyotype of *A. proteus* have faced three seemingly huge obstacles until recently.

First, only metaphase cells, the number of which is very low in the asynchronous laboratory culture of *A. proteus*, are suitable for studying the karyotype. Their number can be estimated roughly using the ratio of the entire cell cycle length to the metaphase of mitosis. The cell cycle in *A. proteus* cultivated under optimal conditions is 40–70 h at 17–25°C (Ord, 1968; Sopina, 1976; Makhlin et al., 1979; Rogerson, 1980; Afon'kin, 1983). According to in vivo observations, the length of the mitotic metaphase does not exceed 5 min (Chalkley and Daniel, 1933). However, this stage is apparently even shorter in reality, since the prophase lasted for about 10 min according to the results of the same study. According to our experimental data, the ratio of prophase and metaphase cells on cytological preparations of the *A. proteus,* strain B is at least 10 : 1. Therefore, the length of these phases of mitosis should differ by approximately an order of magnitude. It follows that, in cytological preparations of an asynchronous culture of amoebas, metaphase cells cannot be found at a frequency of more than 0.05%. It is clear that the synchronization of amoeba culture by the cell cycle would help in this situation, but reliable methods of synchronization have not existed until recently (Yudin, 1990; Makhlin, 1993).

Second, as has been mentioned earlier in the literature (Ord, 1973; Yudin, 1990; Makhlin, 1993; Marakhova et al., 1993) and was confirmed recently by optical imaging (Demin et al., 2015), the linear sizes of mitotic chromosomes in intact cells of *A. proteus* are too small to conduct reliable karyotyping.

Third, a high "yield" of mitotic chromosome spreads on a glass slide is a necessary condition for a comprehensive karyotype study, which is very difficult to achieve in practice because of the specific morphological features of nuclei and the course of the nuclear cycle in *A. proteus*. Vast fragments of the nuclear envelope remain in the metaphase of *A. proteus*. Although this type of mitosis was defined as "semiopen" (Gromov, 1985), in terms of karyotyping, it differs insignificantly from closed mitosis, for which no reliable techniques of chromosome spreading on the surface of a glass slide have been developed so far.

This study aims to resolve the above-mentioned huge obstacles in karyotyping of *A. proteus* by using and adapting several new techniques to the object under study.

In 2013, we proposed a new approach to cell cycle synchronization of amoebas in culture that allowed the frequency of metaphase nuclei in cytological preparations to be increased up to 0.5% (Podlipaeva et al., 2013). In the present study, we used this protocol with minor modifications (see MATERIALS AND METHODS).

As mentioned above, the linear sizes of mitotic chromosomes in intact cells of *A. proteus* are too small for karyotyping. However, it is known that incubation of cells of vertebrate and invertebrate animals in hypotonic saline solutions and spreading of mitotic cells fixated after hypotension on the surface of a glass slide can increase significantly the size of chromosomes. First, the chromosomes elongate in the living cells during chromosome treatment with hypotonic saline solution (Barnitzke et al., 1981); they elongate further during spreading on the surface of a glass slide when fixated cells are hydrolyzed with acetic acid (Claussen et al., 1994; Hliscs et al., 1997). In preparations of differentially stained chromosomes, this stretching is quite sufficient for detecting longitudinal heterogeneity even without additional specific treatments and stains (Comings, 1978; Rønne et al., 1979; Squarzoni et al., 1994).

Finally, we applied a high-pressure squashing technique to remove the nuclear envelope on cytological preparations of amoebas, which interferes with complete spreading of chromosomes. This method was originally developed to improve the resolution of the disk structure of polytene chromosomes of dipteran insects (Novikov et al., 2007; George et al., 2010) and subsequently was successfully applied for obtaining spreads of mitotic chromosomes from invertebrate tissue fragments fixated after hypotonic treatment (Solovyeva et al., 2016). In the latter case, a gradual increase in pressure leads to the rupture and removal of the nuclear membranes and, ultimately, the spreading of chromatin and segregation of individual chromosomes followed by the spreading (linearization) of segregated chromosomes with the exhibition of a chromomere pattern. The pattern of chromomeres was well reproducible, and no signs of destruction of chromosomes (breaks or unclear chromomere pattern) were observed.

MATERIALS AND METHODS

This study was performed using cultured cells of *Amoeba proteus* of strain B from the Amoebae Cultures Collection of the Institute of Cytology, Russian Academy of Sciences (Goodkov et al., 2014). This strain has historically been one of the typical strains of the *A. proteus* species in the Collection and has been characterized comprehensively in a large number of experimental studies (Goodkov et al., 2015). The laboratory culture of amoebas was maintained by standard methods using the Prescott mineral medium (Prescott and Carrier, 1964) at room temperature. Every 48 h, amoebas were fed with the *Tetrahymena pyriformis* GL ciliate (Yudin, 1975).

Synchronization of cells in culture was performed according to a protocol that we developed earlier (Podlipayeva et al., 2013) with minor modifications: 25% solution of bovine serum albumin was used instead of 30% solution of quail-egg protein in the Prescott medium, where we placed amoebas after 2 days of starvation. Under these conditions, all amoebas simultaneously stop moving about, become spherical, and begin to use pinocytosis for feeding. After 1–2 h, amoebas were washed and placed in normal mineral Prescott medium. After 39–41 h of maintaining in this medium at room temperature, the cells were washed with 0.025% solution of Trypsin-EDTA (Sigma-Aldrich, United States) (to prevent their further aggregation) and concentrated in Eppendorf tubes (1.5 mL) by centrifugation at 1500 rpm for 2 min (Eppendorf MiniSpin microcentrifuge, Eppendorf International). After removing the supernatant, the precipitate of cells was vortexed and a hypotonic solution of 2.5 mM KCL in bidistilled water (superQ) was dropped into the resultant suspension under constant shaking. The cells were subjected to hypotonic solutions at room temperature for 20 min with a volume ratio of the cells and hypotonic solution of 1 : (25–30). The cells were then pelleted by centrifugation (2 min, 1500 rpm) and the supernatant was carefully removed. The pellet of cells was resuspended by vortexing, and the resultant suspension was fixated with a mixture of methanol with glacial acetic acid (3 : 1); the fixative was carefully added at constant shaking to avoid cell aggregation. The volume of fixative was adjusted to 1 mL; the cells were centrifuged again and transferred to a fresh portion of fixative. The tubes with the fixated cells were stored in a fridge at –20°C.

For the preparation of spreads of nuclei, we used the technique described in the study by Solovyeva and colleagues (Solovyeva et al., 2016). Glass slides covered with polylysine were used (Thermo Scientific). A concentrated cell suspension of 40–50 μL was placed on the glass surface using a micropipette. Excess of fixative was quickly removed using strips of filter paper. Ten microliters of 50% propionic acid was applied to the cells and immediately thereafter the cells were covered with a 18 × 18-mm coverslip, which was treated with SL-2 silicone (Sigma-Aldrich, United States).

Pressure was applied to squash the cells on the slides via vertical hydraulic vise with pressure manometer to monitor pressure. The pressure was gradually increased using plane-parallel clamps for 90–120 s to until it reached 230 kg/cm^2. The pressure was then dropped; the preparation was taken and was placed

into liquid nitrogen. The coverslip was removed from the frozen preparation using a razor blade. The glass slide with cells was passed through a series of alcohols of increasing strength (50, 70, and 96% ethanol) and then dried. The preparations were placed in a sealed container and stored at –20°C.

The preparations were stained with a DNA-tropic fluorescent dye using freshly prepared solutions of either a YoYo1 iodide bisintercalator (1 μg/mL, Invitrogen Corp., United States) in PBS for 15 min at room temperature or a DAPI one (3 μg/mL, Sigma-Aldrich, United States) in McIlvaine buffer at pH 7.0 for 15 min at room temperature. Before staining with YoYo1, the preparations were treated with RNase A. In some cases, after treatment with RNase A, preparations were further treated with collagenase. Pretreatment of cell preparations with enzymes was performed according to previously described protocols (Khodyuchenko et al., 2012). Preparations were incubated in a solution of RNase A in twofold SSC (saline-sodium citrate buffer, 100 μg/mL, Sigma-Aldrich, United States) for 1 h at 37°C and then washed for 5 min with two replacements of twofold SSC. The preparations were treated with a solution of collagenase (1 mg/mL, Sigma-Aldrich, United States) in PBS for 7 min at room temperature and washed for 5 min in three replacements of PBS. Stained preparations were mounted in a ProLong® Gold antifade (Invitrogen Corp., United States). Images of chromosomal spreads were examined with an Axio Scope A1 microscope (Zeiss, Germany) with a standard digital camera and software for making images. Contrasting and the necessary processing of the images of the chromosomes were performed with the ImageJ and Adobe photoshop CS4 software using previously published techniques (Demin et al., 2011).

RESULTS AND DISCUSSION

Treatment of *Amoeba proteus* with hypotonic solutions at concentrations that are commonly used in cytogenetics for cells of vertebrate and invertebrate animals proved to be ineffective. Only highly dilute hypotonic solutions prepared using bidistilled water exerted the necessary effect on amoebas. A concentration of 2.5 mM KCl was found to be optimal for karyotyping. In addition, the ratio of the volumes of cells to a hypotonic solution was an important factor. Optimal results were obtained when using a ratio of 1 : (25–30). Deviation from this ratio in either direction degraded the quality of chromosome spreads, with either excessive compaction of chromatin or excessive chromatin loosening ("fluffing").

We prepared and analyzed several thousand spreads of nuclei from amoebas subjected to hypotonic treatment. Among the total number of available preparations of mitotic nuclei, 5 metaphase and 18 late prophase–prometaphase chromosome spreads suitable for karyotyping were found. All metaphase nuclei and some of the late prophase-prometaphase nuclei were karyotyped.

It has been established that the basic set of chromosomes in *A. proteus* of strain B is 27 pairs of chromosomes, with each pair being homologous in the pattern of chromomeres (bands) (Fig. 1). The obtained metaphase karyograms did not differ from each other by number and morphology of individual chromosomes; hence, the presented karyogram in this study can be considered a typical karyogram (Fig. 2).

For differential staining of chromosomes, we used two DNA-tropic fluorochromes with different targets. The DAPI dye associates with the minor groove of DNA and has a high affinity to AT-pairs of nucleotides (Kapuscinski, 1995). It produces a differential chromosome-staining pattern with longitudinal heterogeneity of the chromosomes in the distribution of both AT-rich chromatin loci and areas with moderate contents of AT-pairs, but significantly different levels of chromatin packing. The YoYo1 iodide dye is a DNA bisintercalator and is not a nucleotide-specific agent (Rye et al., 1992). It can produce a differential chromosome staining pattern only in the presence of longitudinal chromosome heterogeneity in chromatin packing. Theoretically, these dyes can yield a different pattern of differential chromosome staining, but this does not occur in *A. proteus* (Fig. 2). The chromomere pattern during staining with these dyes was very similar, though not quite identical. This means that the chromomeric and interchromomeric bands of the chromosomes of an amoeba do no differ significantly in content of AT-base pairs. The pattern of YoYo1-staining of individual chromosomes of an amoeba did not reveal additional bands compared to the pattern after DAPI-staining, which means that the chromosomes of *A. proteus* of strain B do not contain DAPI-negative GC-rich bands.

Overall, in the pattern of DAPI and YoYo1 banding of chromosomes of *A. proteus*, strain B was found to be well reproducible. In our opinion, pretreatment with enzymes had a positive effect on the resolution of the chromosome pattern, especially in the case of sequential pretreatment with RNase and collagenase (Fig. 2).

According to optical tomography, the size of mitotic-prophase nuclei of *A. proteus* is significantly reduced by the time of the metaphase onset (Demin et al., 2015), which is apparently due to the elimination of so-called "excessive DNA" (Afon'kin, 1983; Makhlin, 1987, 1993; Afon'kin, 1989) by the end of the mitotic prophase as a result of its extrusion from the nucleus (Demin et al., 2015). In other words, according to the results of the present study, all the "extra" chromosomes have already been eliminated from the nucleus of an amoeba by the onset of the

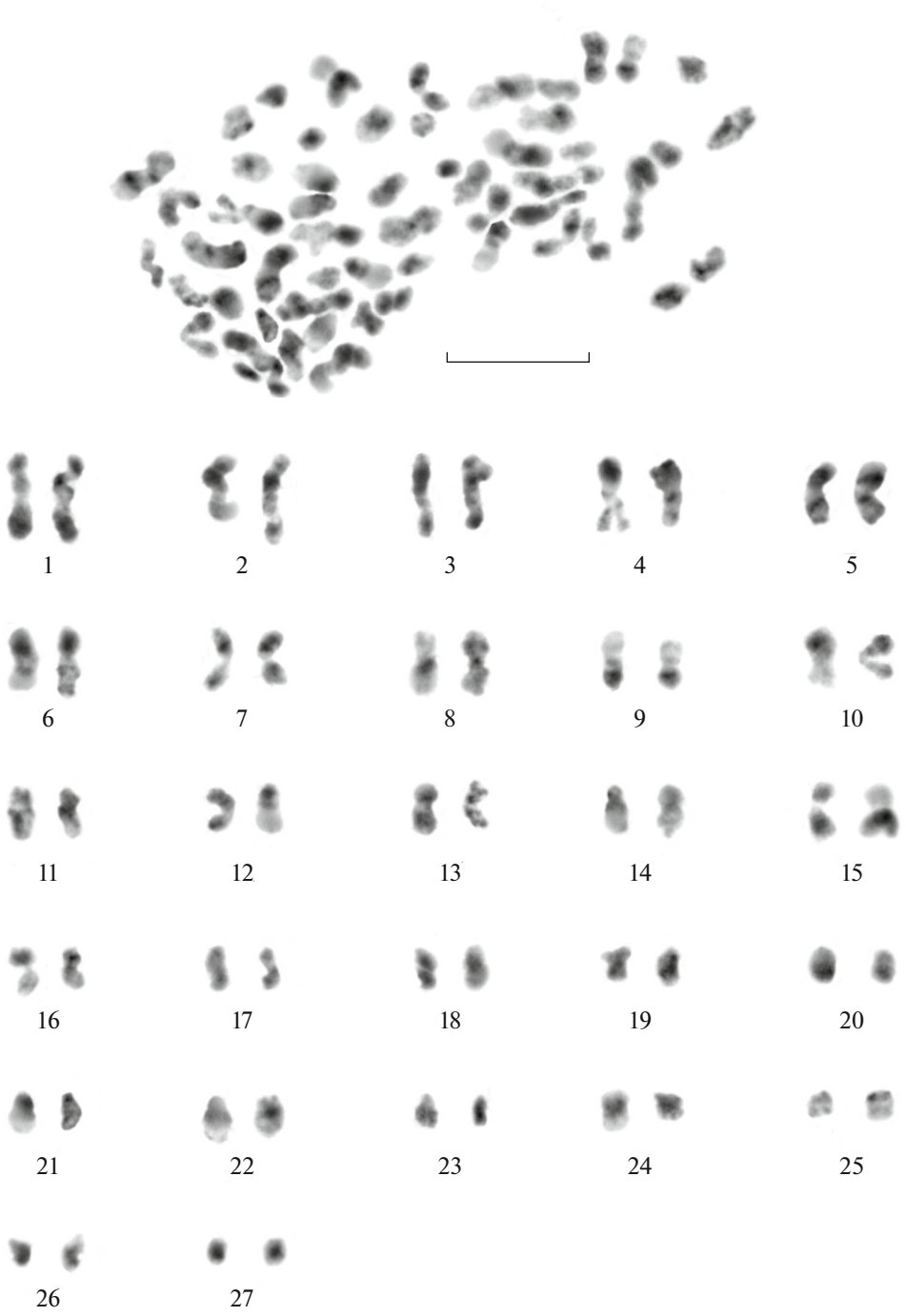

Fig. 1. Negative image of a typical spread of metaphase chromosomes and a karyogram of *A. proteus*, strain B. Staining with a solution of YoYo1 iodide after pretreatment with RNase A. Scale bar: 10 μm.

metaphase. However, in the late prophase, in some cases, individual "extra" chromosomes can be still found, which have a chromomeric pattern of the basic set. Most often, though not always, these are the smallest chromosomes of the set.

Thus, the results of this study fully support our earlier hypothesis (Demin et al., 2015) that the basic chromosome number of the species *Amoeba proteus* is significantly smaller than it has been to be considered so far (Ord, 1973; Yudin, 1990; Makhlin, 1993;

Fig. 2. Bank of negative images of YoYo1- and DAPI-banded individual chromosomes of *A. proteus,* strain B, composed of five different spreads of mitotic cells. The figures indicate the numbers of groups of individual chromosomes. The asterisk points to YoYo1-banded chromosomes pretreated with RNase A; the lattice points to YoYo1-banded chromosomes pretreated with RNase and collagenase. Scale bar: 10 μm.

Marakhova et al., 1993). It is no doubt the case that the nuclear cycle of *A. proteus*, at least in the strain of amoebas under study, necessarily involves the elimination of "extra" chromosomes by extrusion, which can continue up to the late prophase of mitosis. A detailed description of this phenomenon is beyond the scope of this work and will be presented later in a separate report.

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