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Programmable DNA Cleavage by Cyanobacterial Argonaute Proteins

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Abstract—Argonaute proteins are an evolutionarily conserved family of proteins capable of recognizing and cleaving specific nucleic acid sequences using complementary guide molecules. Eukaryotic Argonautes play a key role in RNA interference by utilizing short RNAs of various classes to recognize target mRNAs. Prokaryotic Argonautes are much more diverse and most of them recognize DNA targets. The search for new Argonautes that would be active under varying conditions is important for both understanding their functions and developing new tools for genetic technologies. Many previously studied Argonautes exhibit low activity at low and moderate temperatures. To overcome this limitation, we isolated and studied two Argonaute proteins from psychrotolerant cyanobacteria, CstAgo from *Cyanobacterium stanieri* and CspAgo from *Calothrix* sp. Both proteins use short DNA guides to recognize and cleave DNA targets. CstAgo displayed no specificity for the 5′-end structure of the guide, while CspAgo demonstrated a weak preference for the 5′-terminal nucleotide. CstAgo was highly active and capable of cleaving single-stranded DNA at temperatures from 10 to 50°C. CspAgo was more cold-sensitive but cleaved double-stranded plasmid DNA using specific guides. Therefore, the studied proteins can be potentially used for DNA manipulations under a wide range of conditions.

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INTRODUCTION

The genes for Argonaute proteins were discovered in the late 20th century, shortly followed by elucidation of the RNA interference (RNAi) mechanism. It was shown that complexes of Argonautes with short guide RNAs play a central role in recognizing target mRNA molecules [1, 2]. Argonautes have been found in almost all eukaryotes. The role of RNAi includes regulation of gene expression, suppression of transposition of mobile elements in the germline, and defense against RNA viruses [3]. RNAi studies have led to the development of efficient tools for suppressing expression of target genes, which are currently used in analysis of gene functions and therapy [4].

About 12 years ago, researchers have turned their attention to prokaryotic Argonautes (pAgos) [5, 6], which had previously been identified by bioinformatics methods [7] and used for structural studies of RNAi mechanisms [8, 9]. pAgos are present in approximately 10% bacterial genera and 30% archaeal genera, and they are much more diverse in structure than their eukaryotic homologs. pAgos can be divided into three major groups depending on the presence of specific domains and catalytic activity: long-A pAgos (active), long-B pAgos (inactive), and short pAgos (inactive) [7, 10]. Inactive Argonaute proteins are often encoded in the same operon with additional partner proteins. Initially used as models to understand the functions of eukaryotic proteins, pAgos are actively investigated today to elucidate their biochemical, structural, functional properties and to allow their application in biotechnology.

Long Argonautes consist of six domains, N, L1, PAZ, L2, MID, and PIWI. They have a two-lobe structure, with one lobe consisting of the N and PAZ domains and the other consisting of the MID and PIWI

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domains, with nucleic acids positioned between them. The PIWI domain is structurally related to RNase H-like proteins; it contains the DEDX catalytic tetrad (where X is D, H, K, or N), coordinates divalent cations, and is necessary for catalyzing the cleavage of the target [11]. The N-terminal domain is the least conserved and is important for positioning the guide-target duplex. The PAZ domain forms a pocket for binding the 3′-end of the guide molecule in the binary complex before the target binding. The MID domain contains a pocket for binding the 5′-end of the guide. Structural studies have shown that the 5′-terminal nucleotide does not form complementary interactions with the target and can be specifically recognized by Argonautes in the MID pocket [12]. For example, RsAgo from *Rhodobacter sphaeroides* (now *Cereibacter sphaeroides*) and many eukaryotic Argonautes preferentially bind guides with the 5′ terminal U nucleotide, TtAgo from *Thermus thermophilus* binds guides with the 5′ C nucleotide, while some proteins have no specificity for the 5′-end of the guide [5, 6, 11-14]. The presence at the 5′ end of a phosphate group, which interacts with conserved amino acid residues of the MID pocket and a Mg^{2+} ion bound in the pocket, is important for guide binding by most Argonautes [11, 15, 16]. The binding of suboptimal guides occurs with a lower affinity and may lead to a shift in the target cleavage site due to the reduced complex stability [15].

pAgos efficiently bind guides of 16 to 24 nucleotides *in vitro* [17, 18] and typically use guides of the same length *in vivo* [12, 16]. It has been established that most catalytically active pAgos use single-stranded DNA guides and recognize DNA targets, acting as guide-dependent endonucleases and cleaving the target at a strictly defined position (similar to eukaryotic proteins) between the 10th and 11th nucleotides from the 5′ end of the guide [6, 16, 19-23]. Later, additional groups of pAgos have been discovered that can recognize and cleave DNA targets using RNA guides [24, 25], RNA targets using DNA guides [15, 26], as well as RNA targets using RNA guides [27]. Some Argonauts are able to use different combinations of guides and targets (both DNA and RNA) *in vitro*, although with lower efficiency than optimal guides and targets [23]. In the case of catalytically inactive Argonautes, additional effector nucleases encoded in the same operon may be involved in target DNA processing [5, 7, 10, 28, 29].

Studies of first bacterial Argonautes suggested that these proteins modulate the amount of invader DNA in cells by affecting the processing of plasmids and decreasing the transformation efficiency [5, 6]. Direct evidence of pAgo functions in bacterial immunity was obtained later in a heterologous *E. coli* system for clostridial Argonaute, which was shown to suppress viral infection [30]. The antiphage activity has been then demonstrated for several long and short Argonautes [18, 28, 31-33]. Some Argonautes have the ability to process double-stranded DNA at random sites in the absence of guide molecules (the so-called ‘chopping’ activity). It is assumed that this process can generate new guides during initial recognition of invader DNA in the cell [34].

Initially, most studied Argonautes were isolated from thermophilic bacteria. Since these proteins were easy to be crystallized, this allowed to combine the data on the protein structure and their properties [35]. However, a potential possibility of using Argonautes for genome editing in eukaryotic cells has stimulated the investigation of proteins from mesophilic bacteria [16, 22]. These proteins have several advantages compared to Cas nucleases, including smaller size, lack of requirement for a PAM motif, and use of short DNA guides (see reviews [12, 36]). Successful application of Argonautes in genome editing of bacterial cells has already been demonstrated [37, 38]; optimization of Argonaute-based tools for the use in eukaryotic cells is currently ongoing.

pAgos have already been used for developing tools for targeted DNA cleavage *in vitro*, similar to restriction endonucleases [39], as well as for detection of nucleic acids (short RNAs, rare DNA variants, nucleic acids of pathogens, RNA and DNA modifications) [12, 36, 40-44]. Many of these methods are based on the ability of Argonautes to bind and cleave single-stranded targets *in vitro*. However, the processing of double-stranded DNA and genome editing depend on the ability of Argonautes to cleave both DNA strands; such activity requires the presence of two guides and local unwinding of the two DNA strands before their cleavage [16, 22, 23]. Genome editing in eukaryotic cells also requires proteins with a temperature optimum close to physiological temperatures. DNA supercoiling, replication, transcription, and binding of histones and regulatory proteins can significantly alter the accessibility of target sites in the genome. Therefore, to create optimal genomic editors, their activity must be tested *in vitro* and *in vivo* under a wide range of conditions.

The search for new catalytically active bacterial Argonautes capable of cleaving double-stranded DNA under physiological conditions without auxiliary proteins, as well as development of methods for their additional activation, are among the most important tasks in the field of Argonaute research. Some previously tested Argonautes from mesophilic bacteria have been found to cleave double-stranded DNA at temperatures above 50°C but to have low activity at physiological temperatures [16, 22, 23]. One way to solve this problem is to study new representatives

of Argonaute proteins from psychrophilic and psychrotolerant bacteria presumably capable of functioning at lower temperatures.

MATERIALS AND METHODS

Cloning and expression of Argonaute genes. The amino acid sequences of Argonaute proteins from cyanobacteria *Cyanobacterium stanieri* LEGE 03274 (WP_193799343.1) and *Calothrix* sp. PCC 7103 (WP_019494780.1) were codon-optimized for expression in *Escherichia coli* cells using the IDT Codon Optimization Tool and obtained by chemical synthesis (Cloning Facility, Moscow) as three fragments overlapping by 20 nt. The full-length genes were cloned into the pET28b+ vector using the Gibson assembly method with a N-terminal His_6 tag and under the control of the *T7* promoter. Gene sequences are provided in the Supplementary Materials (Online Resource 1).

Purification of Argonaute proteins. The proteins were expressed in *E. coli* BL21(DE3) cells transformed with the corresponding vectors. To isolate CstAgo, the cells were grown in a shaker incubator in LB medium containing 50 μg/mL kanamycin and 0.2% glucose to OD_{600} = 0.3 at 30°C, then cooled in an ice bath, and transferred to 18°C. Protein synthesis was induced with 0.2 mM IPTG, and the cells were grown for 20 h with constant shaking at 230 rpm. To obtain CspAgo, the cells were grown in LB medium containing 50 μg/mL kanamycin to OD_{600} = 0.3 at 37°C, then cooled in an ice bath, and transferred to 16°C. IPTG was added to 0.2 mM, and the cells were grown for 20 h. The cells were collected by centrifugation and stored at −20°C.

The cell pellet obtained from 2 L of culture was thawed and resuspended in buffer A (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole, 5% glycerol), passed three times through a high-pressure continuous flow cell disruptor (Constant Systems, UK) at a pressure of 30 kpsi, and 1 mM PMSF was added to the lysate. The lysate was centrifuged twice at 30,000*g* for 15 min at 4°C in a Hitachi CR22N centrifuge (rotor R21A) (Japan). The supernatant was applied on a HisTrap FF column (GE Healthcare, USA). The column was washed with the buffer containing 20 mM and 49 mM imidazole for CspAgo or 92 mM imidazole for CstAgo, elution was performed with 260 mM imidazole. The resulting fractions were analyzed by Laemmli electrophoresis followed by Coomassie G-250 staining. Fractions containing expressed Argonaute proteins were diluted 10-fold with a buffer containing 10 mM HEPES-KOH (pH 7.0), 0.2 M NaCl, and 5% glycerol, applied on a HiTrap Heparin HP column (GE Healthcare), and eluted with a stepwise NaCl gradient (200, 320, 440, 680, and 1000 mM). Fractions containing the target proteins were concentrated using Amicon Ultra 50 kDa (Millipore, USA). HEPES-KOH (pH 7.0) and EDTA were added to the protein samples to 20 and 0.5 mM, respectively; the samples were mixed with glycerol at 1 : 1 ratio and stored at −20°C (for several months) or −70°C after freezing in liquid nitrogen (for longer periods of time). Protein concentration was determined using Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific, USA).

Analysis of Argonaute activity *in vitro*. Synthetic DNA and RNA molecules (Evrogen, Russia; DNK-Sintez, Russia) were used as guides and targets in the cleavage reactions (see Table S1 in the Online Resource 1 for the sequences). RNA and DNA guides were 5′-phosphorylated with T4 polynucleotide kinase (New England Biolabs, USA) in a buffer solution containing 20 mM HEPES-KOH (pH 7.0), 100 mM NaCl, 10% glycerol, and 5 mM $MgCl_2$. To study the effect of divalent cations on the cleavage activity, $MgCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, and $MnCl_2$ at the concentrations of 0.5 and 5 mM were used. The purified proteins (final concentration, 500 nM) were incubated with the guide (500 nM) at 37°C for 15 min, and the target (100 nM) was added. The reaction was carried out at 37°C for 15 min for CstAgo and 30 min for CspAgo. When studying the effect of temperature on the Argonaute activity, the proteins were loaded with a standard phosphorylated G-guide for 15 min at 37°C. Then, aliquots of the reaction mixture were incubated at temperatures from 10 to 50°C, after which the target was added, and the reaction was carried out for 30 min. The reaction was stopped by adding an equal volume of denaturing loading buffer (8 M urea, 100 mM EDTA, 2× TBE buffer, 0.005% xylene cyanol, and 0.005% bromophenol blue) after the time indicated in the figure captions.

DNA or RNA molecules were separated electrophoretically in a denaturing 19% polyacrylamide gel (acrylamide : bisacrylamide ratio, 19 : 1; 7 M urea) in TBE buffer in a MiniPROTEAN Tetra Cell system (Bio-Rad, USA) at 300 V for 1 h 15 min. Nucleic acids in the gel were visualized by staining with SYBR Gold (Invitrogen, USA), and the gel was scanned in the appropriate channel on a Typhoon FLA 9500 scanner.

To determine the reaction kinetic parameters, the targets were labeled with P^{32} at the 5′-terminal position using T4 polynucleotide kinase and γ-P^{32}-ATP (New England Biolabs, USA). The position of the radioactive label in the gel was determined using a radio-sensitive screen (GE Healthcare) and a Typhoon FLA 9500 scanner (GE Healthcare Life Sciences). The results were analyzed using the ImageQuant program (GE Healthcare) and GraphPad Prism 9.

To analyze the cleavage of plasmid DNA, the pJET1.2 plasmid with short inserts was used (see Table S1 in the Online Resource 1 for the sequences).

CspAgo or CstAgo (500 nM) was mixed with 5′-phosphorylated guide DNA (500 nM) in the reaction buffer and incubated at 37°C for 10 min. When 2 or 4 guides were used in the same reaction, they were loaded into the Argonaute protein independently and then mixed. Next, plasmid DNA (2 nM) was added and the samples were incubated for 10 or 40 min at 37 or 50°C. The reaction was stopped by adding 1 μL of Proteinase K (Thermo Fisher Scientific); the samples were incubated for 20 min at 25°C and then loaded on 1.2% agarose gel containing 0.5× TBE. SYBR Gold was added to the samples for visualization. Electrophoresis was carried out in 0.5× TBE in a SE-2 chamber (Helicon, Russia) at 100 V.

RESULTS

Cyanobacterial Argonautes. To search for Argonautes from psychrophilic bacteria, we used a previously constructed Argonaute phylogenetic tree with the corresponding list of strains [10]. The search was conducted among Argonaute proteins of the long-A group, with a predicted full active site in the PIWI domain. The temperature optima of bacterial strains in this group were manually determined based on their description in microbiological collections. Two proteins from cyanobacteria were selected: WP_019494780.1 (CspAgo) from *Calothrix* sp. PCC 7103 and WP_193799343 (CstAgo) from *Cyanobacterium stanieri* LEGE 03274 (growth temperature 19°C, according to the LEGE culture collection catalog). Since these bacteria inhabit cold water, we assumed that their Argonautes are adapted to functioning at lower temperatures. However, by the time of writing this article, the WP_019494780.1 protein had been removed from the NCBI database. Its closest homolog with 90% identity is Argonaute protein RIVM261_088880 from *Rivularia* sp. IAM M-261 [45] (strain isolated in Bangkok from a cement wall). The positions of the studied cyanobacterial Argonautes on the phylogenetic tree is shown in Fig. 1a.

The genes coding for CspAgo and CstAgo were optimized for expression in *E. coli*, cloned under the control of an inducible promoter, and expressed as described in the Materials and Methods. CstAgo was found to be toxic under standard expression conditions, so the induction conditions for this protein were modified (see Materials and Methods). The proteins were isolated from the cells disrupted under high pressure, which allowed to preserve significant amounts of CspAgo and CstAgo in the soluble form, as has been previously found for other Argonautes.

Although significant amounts of CstAgo and CspAgo were present in the cell lysate after centrifugation, the proteins were also found in the precipitate, indicating possible formation of inclusion bodies. The proteins were purified in two steps using nickel-affinity and heparin chromatography to almost complete absence of impurities according to staining with Coomassie G-250, and then concentrated (see Fig. 1c for CspAgo).

Guide specificity of CstAgo and CspAgo. To determine the activity of the purified proteins, in particular, whether they can cleave DNA, their specificity for guides and targets was studied in *in vitro* tests. Both Argonautes have a standard MID pocket structure and contain conserved residues that interact with the 5′-phosphate group of the guide, so experiments were conducted with 5′-phosphorylated guides. For this, the proteins were loaded with RNA or DNA guides of identical length and sequence. We used 18-nt guides, as this length was found to be optimal for most previously studied pAgos (see Introduction). After formation of the binary complex, a 50-nt target (RNA or DNA) with a central region complementary to the guide was added to the reaction (Fig. 2a). The reaction was carried out for 4 guide–target combinations: DNA–DNA, DNA–RNA, RNA–RNA, RNA–DNA (G-guides and G-targets; the letter corresponds to the 5′-terminal nucleotide of the guide, see Online Resource 1). The guide was incubated with the Argonaute at a 1 : 1 ratio at a concentration of 500 nM, which was significantly higher than the guide binding constants measured for related proteins (nanomolar and subnanomolar ranges) [15, 16, 18, 46]. The reaction was carried out for 15 or 30 min and the reaction products were separated by electrophoresis in denaturing PAAG and stained with SYBR Gold for nucleic acid detection (Fig. 2b). It can be seen that the target cleavage with the formation of 23 and 27 nt products (corresponding to the lengths of synthetic marker oligonucleotides) occurred only in the case of DNA guide–DNA target combination (Fig. 2b, lane *1*). The absence of activity with other guide–target combinations might be explained by much less efficient binding of non-optimal guides and targets by the Argonaute proteins and altered position of these molecules in the active site [6, 16, 18]. Hence, we demonstrated that the purified proteins possess the desired activity and can be used in further experiments to analyze the optimal conditions for DNA cleavage.

Some Argonautes have a strict specificity for the 5′-end guide nucleotide due to the structural features of the MID domain pocket, in which this nucleotide is bound (see Introduction). However, the absence of such specificity is desirable for the potential application of Argonautes for genome editing, as it limits the choice of guides. To check the specificity of the purified Argonaute proteins to the 5′-terminal nucleotide of the guide, we carried out cleavage reactions with guides containing different nucleotides at the 5′-end

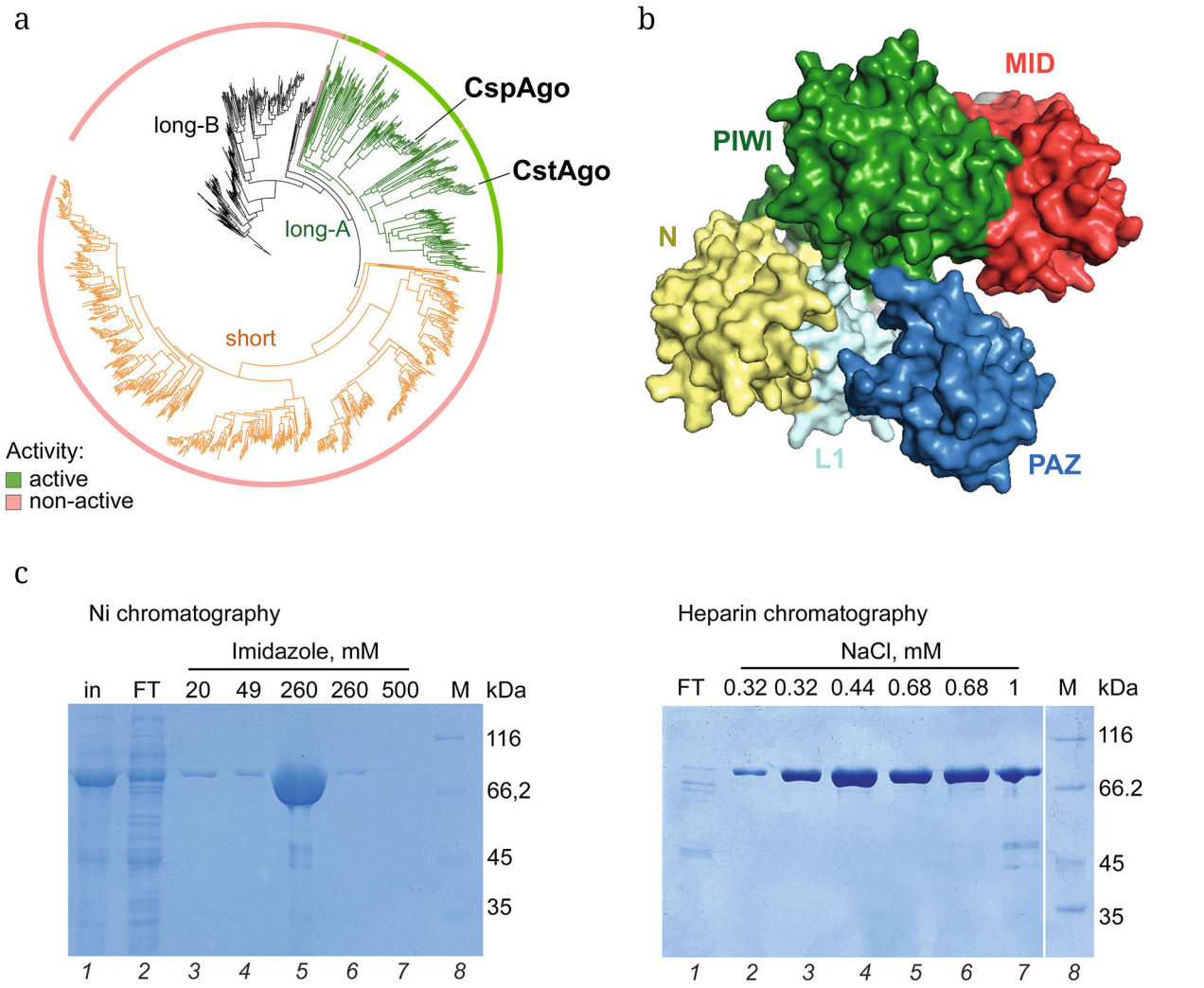

Fig. 1. Cyanobacterial Argonautes CspAgo and CstAgo. a) Phylogenetic tree of Argonautes. The branches are colored according to three protein groups: long-A (green), long-B (black), and short (orange). The presence and absence of catalytic activity are indicated in the outer circle by light pink and green, respectively. b) The three-dimensional model of CstAgo with indicated domains (created by AlphaFold). c) Purification of CspAgo. Electrophoretic analysis in 10% PAAG of fractions obtained after nickel-affinity and heparin chromatography steps: 'in', protein sample applied to the column; FT, flowthrough; M, molecular weight markers.

and corresponding DNA targets: A-guide/A-target, G-guide/G-target, C-guide/C-target, and T-guide/T-target. In all cases, the guides were phosphorylated at the 5′-end and fully complementary to the target.

The cleavage of the target was carried out for 3 and 10 min to observe a possible effect of the 5′-terminal nucleotide in the guide on the rate of target cleavage. CstAgo did not display any preference for the 5′-terminal nucleotide of the guide. CspAgo cleaved the targets in the presence of A, T, or G at the 5′-end of the guide with similar efficiencies, but the presence of C at the 5′-end of the guide somewhat reduced the efficiency of cleavage (Fig. 2c).

To determine the preference of CstAgo and CspAgo for catalytic metal ions, the cleavage reaction was carried out with various divalent metal cations taken at two concentrations, 0.5 mM and 5 mM. The results of the experiment (Fig. 3a) showed that CstAgo was active with both concentrations of Mg^{2+} (0.5 and 5 mM), as well as in the presence of 5 mM Mn^{2+}. In contrast, CspAgo exhibited activity at both concentrations of Mn^{2+} and only at 5 mM Mg^{2+}. The pattern of target cleavage by both proteins in the presence of both cations was the same. Cu^{2+}, Zn^{2+}, Co^{2+} did not act as catalytic cations. Therefore, both studied proteins were active at the physiological concentration of magnesium ions.

Temperature dependence of Argonaute activity and the rate of target cleavage. To check whether CstAgo and CspAgo can function at physiological temperatures, we studied the temperature dependence of target cleavage by these proteins (Fig. 3b).

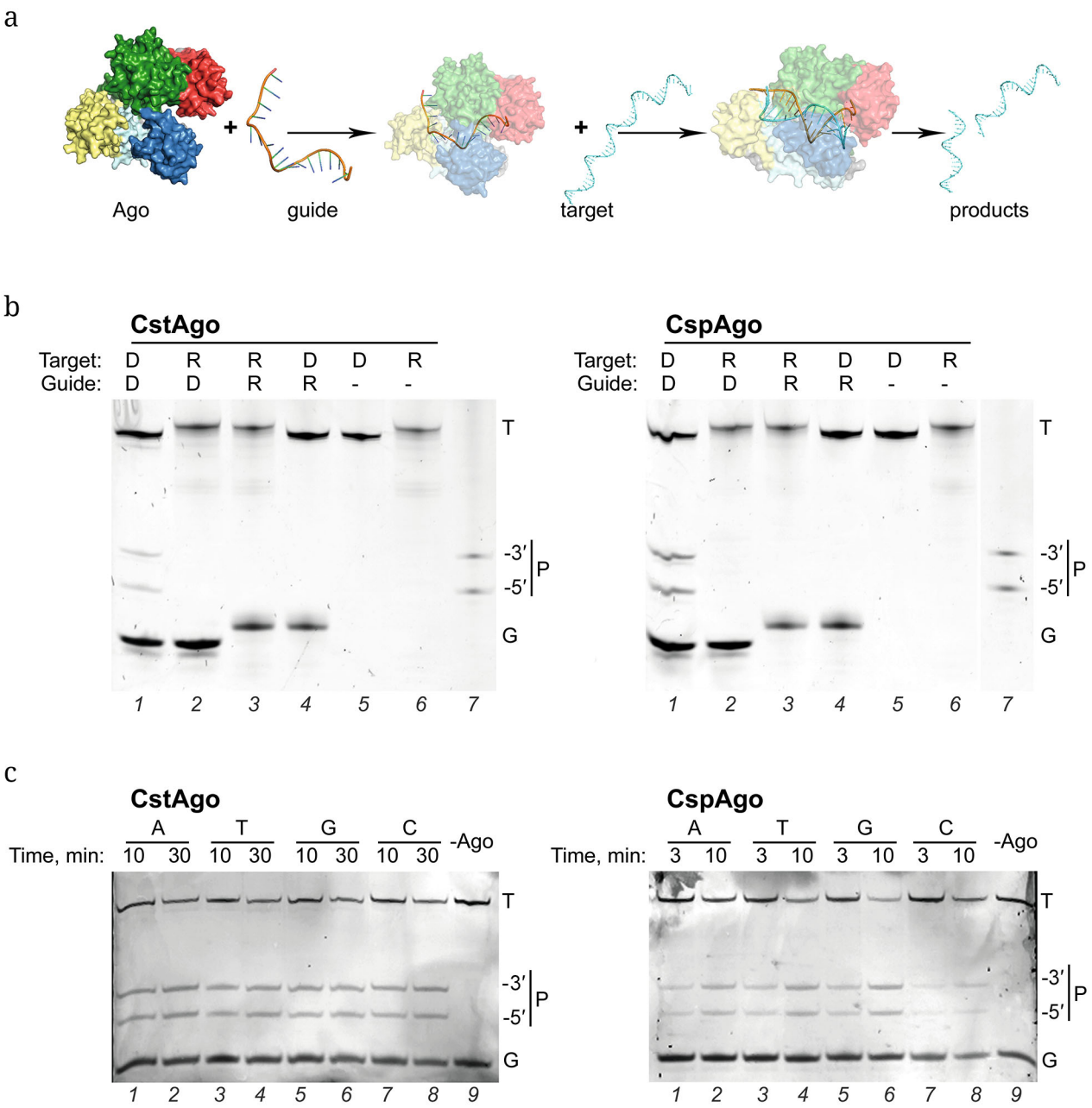

Fig. 2. Specificity of CstAgo and CspAgo toward guides and targets. a) The reaction scheme showing the Argonaute protein, guide, target and the reaction products. b) Determination of the specificity of CstAgo and CspAgo toward DNA (D) and RNA (R) guides (G) and targets (T). P, oligonucleotides with the lengths corresponding to the expected lengths of products of the target cleavage between the 10th and 11th nucleotides of the guide. The cleavage reaction was carried out for 30 min. c) Dependence of the CstAgo and CspAgo nuclease activity on the 5′-terminal nucleotide in guide DNA. Reactions were carried out at 37°C with 5′-phosphorylated guides with different 5′-terminal nucleotides and complementary DNA targets.

CstAgo was active within the temperature range from 10 to 50°C, and the ratio of the reaction products to the unreacted target increased with the temperature increase. CspAgo had a different profile of temperature dependence. It was active only above 20°C, and even at 30°C the amount of cleaved target remained low; the highest activity was observed at 50°C. Such temperature dependence is similar to that found for previously studied Argonautes from mesophilic bacteria [47, 48]. At the same time, both CstAgo and CspAgo are active at 37°C and are therefore promising candidates for use in cells at moderate temperatures. The ability of CstAgo to exhibit activity at 10°C makes it the most cold-resistant Argonaute protein among a large number of previously studied representatives of this group [16, 22, 23, 47, 48].

Another important characteristic of potential genome editors is the rate of target cleavage. For a more

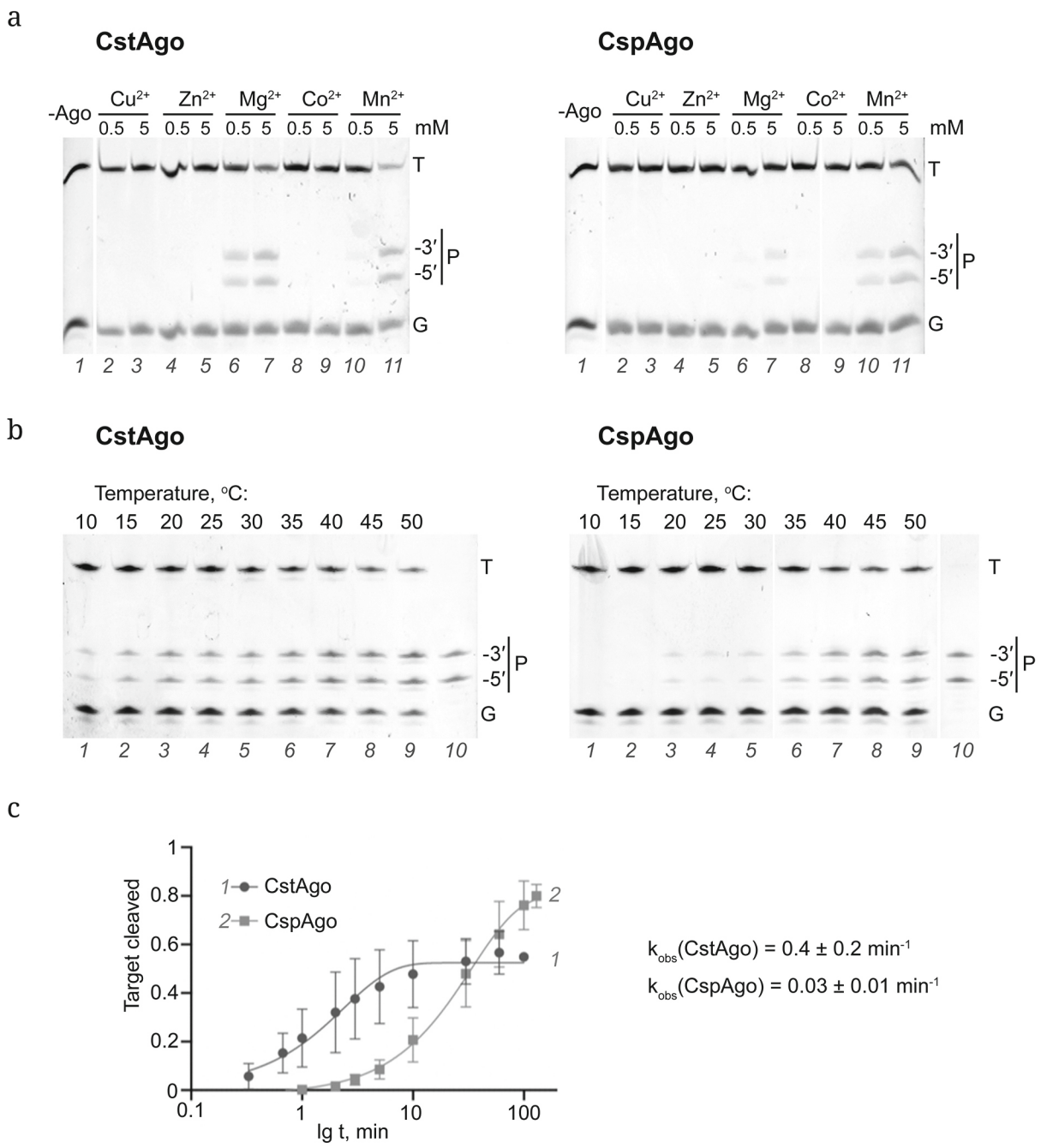

Fig. 3. Effect of reaction conditions on the activity of CstAgo and CspAgo. a) Dependence of the Argonaute nuclease activity on divalent cations. The reaction was carried out for 15 min with CstAgo and 30 min with CspAgo; T, target; G, guide; P, products. b) Dependence of the Argonaute activity on reaction temperature. The reaction was carried out for 30 min at the indicated temperatures. c) Kinetics of DNA target cleavage by CstAgo and CspAgo. The reaction was carried out at 37°C. For each time point, the ratio of the shortened reaction product to the full-length target was determined. To calculate the observed rate constants (k_{obs}), the reaction kinetics were approximated by a first-order kinetics equation. The data are shown as means ± standard deviations for three independent experiments.

accurate measurement of this parameter, we used a DNA target radioactively labeled at the 5′-end. The reaction was carried out at 37°C, and the ratio of the 5′-labeled cleavage products to the total amount of labeled DNA was measured at different time intervals (Fig. 3c; observed rate constants k_{obs} are shown on the right). CstAgo cleaved the target approximately 10 times faster than CspAgo; CstAgo cleaved half of the target within several minutes vs. CspAgo that required tens of minutes (Fig. 3c). Therefore, CstAgo has one of the highest rates of target cleavage among the studied Argonautes, comparable to the previously described KmAgo [23].

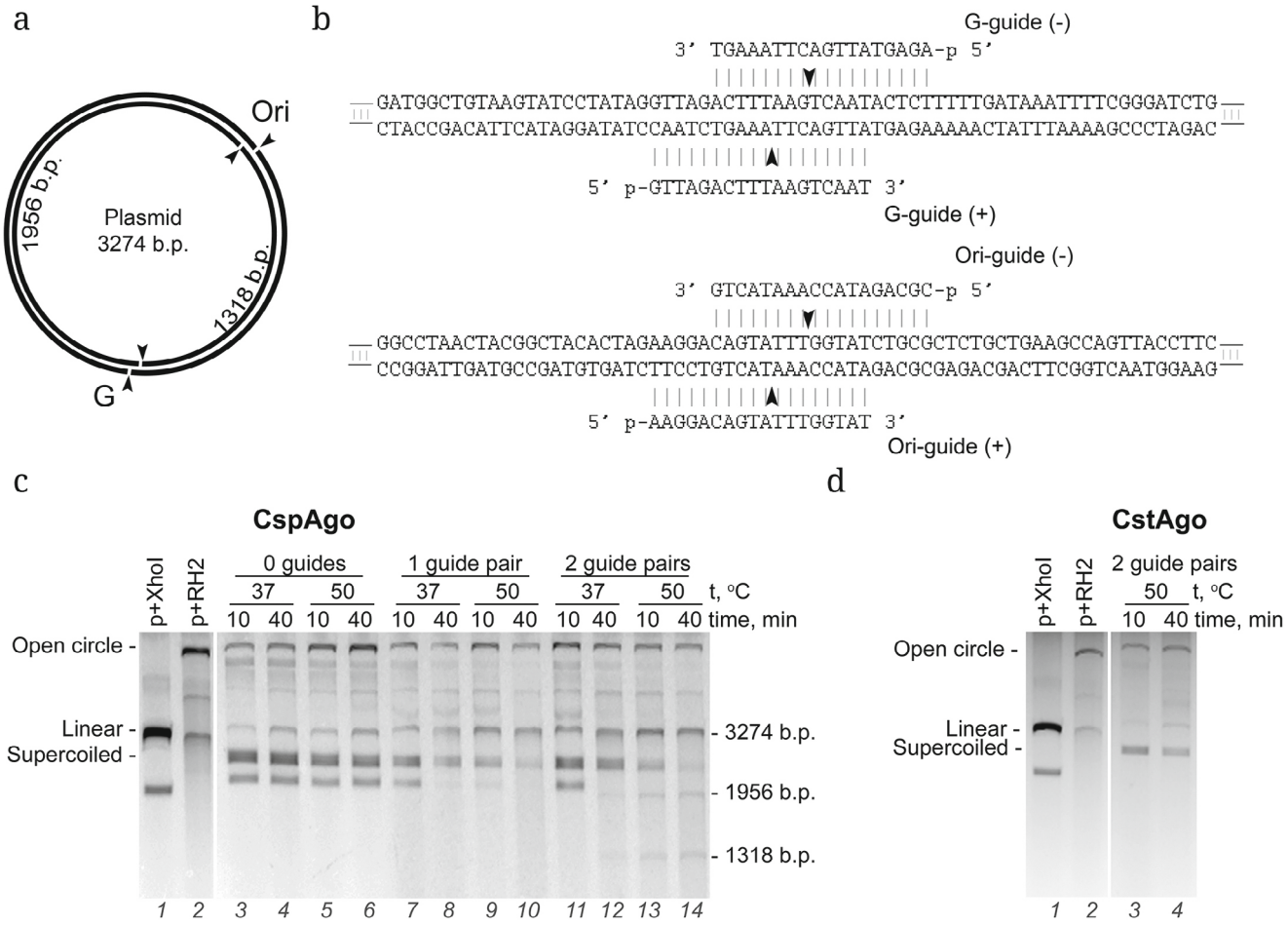

Fig. 4. Cleavage of plasmid DNA by Argonautes. a) Scheme of plasmid DNA cleavage using two pairs of DNA guides. b) Sequences of guides and targets. The arrows show the cleavage sites. c) Cleavage of plasmid DNA by CspAgo. Electrophoresis of cleavage products in native agarose gel stained with SYBR Gold. Plasmid treated with XhoI restriction endonuclease (lane *1*, p+XhoI) and RNase H2 (lane *2*, p+RH2) was used as a control. The reaction was carried out with one or two pairs of guides for the indicated time at 37°C or 50°C. The products of DNA hydrolysis at the indicated sites (1956 bp and 1318 bp) are shown. d) Cleavage of plasmid DNA by CstAgo. The reaction was carried out at 50°C for 10 and 40 min.

Plasmid DNA cleavage. Next, we tested the activity of CspAgo and CstAgo Argonautes toward the plasmid DNA, since the ability to cleave double-stranded DNA may be important for genome editing *in vivo*. The hydrolysis of plasmid DNA by Argonautes was studied using a previously developed pJET vector containing an insert of the target DNA. Two pairs of guides were selected for the sites located 1.3 kb apart: G-guide (+) used in the experiments on single-stranded DNA and its complementary G-guide (–) in the DNA insert region, and the Ori-guide (+) and its complementary Ori-guide (–) in the plasmid replication origin region. Complementary guides were offset relatively to each other by 5 nucleotides so that cleavage of DNA by two Argonautes at the same locus would lead to the formation of 5 nt sticky ends (Fig. 4a and b). Successful cleavage of plasmid DNA by Argonautes in this system would led to the formation of two linear products 1956 and 1318 bp long (Fig. 4a).

CstAgo and CspAgo were independently loaded with four guides and incubated with plasmid DNA in the reaction buffer at 37°C or 50°C for 10 or 40 min. The plasmid cleaved at a unique XhoI site (linear DNA) and plasmid treated with RNase H2 (relaxed circular form) were used as controls. The relative positions of all plasmid forms and the products of the control reaction with CbAgo (which specifically cleaves plasmid DNA [16, 22]) are shown in the supplementary data (Online Resource 2). It was found that in the absence of guides (Fig. 4c, lanes *3-6*), CspAgo did not affect the distribution of plasmid DNA forms at both temperatures, indicating the absence of non-specific (chopping) activity under these conditions. When one pair of the guides was added (Fig. 4c, lanes *7-10*), one of the bands corresponding to the supercoiled plasmid disappeared (the lower band on the gel), which could be seen after 40 min of incubation at 37°C and at both time points at 50°C.

When two pairs of guides were used, two linear cleavage products of the expected length were observed after 40 min of incubation at 37°C (lane *12*) and at both time points at 50°C (lanes *13* and *14*). Therefore, the cleavage of the plasmid DNA by CspAgo occurred more efficiently at 50°C than at 37°C; however, this efficiency did not reach 100% under the tested conditions. At the same time, CstAgo was unable to cleave the plasmid DNA in the presence of two pairs of guides either at 37°C or at 50°C (Fig. 4d; results obtained at 50°C are shown). Hence, of the two studied Argonautes, only CspAgo cleaved plasmid DNA using specific guides.

DISCUSSION

Argonautes are programmable nucleases that recognize target nucleic acids using short guide molecules; hence they can be potentially used in biotechnological applications along with CRISPR-Cas nucleases [12, 36]. Studied pAgos have diverse catalytic properties and can use DNA or RNA guides and recognize DNA or RNA targets. However, an important limitation of the previously studied Argonautes is their low activity at physiological temperatures, especially toward double-stranded DNA. Many Argonautes have been isolated from thermophilic bacteria and archaea, so they have a high thermostability but are unable to cleave DNA at low or moderate temperatures [6, 19, 34, 35]. In order to expand the range of available Argonautes, we purified and investigated the properties of two Argonautes, CstAgo and CspAgo, from psychrotolerant cyanobacteria, whose temperature optimum for growth is lower than that of most mesophilic bacteria previously used as a source of Argonaute proteins [47, 48]. Due to the difficulties in obtaining specific bacterial strains, the genes for the studied proteins were constructed from synthetic DNA fragments, cloned into expression vectors and used for isolation of highly purified preparations of the expressed proteins.

The main goal of this work was to study the efficiency of DNA cleavage *in vitro* by the new cyanobacterial Argonautes to assess their activity under physiological conditions. The experiments showed that both CstAgo and CspAgo are DNA guide-dependent DNA nucleases that have no preference for the 5′-terminal nucleotide of the guide and are active in the presence of magnesium and manganese cations. At the same time, CspAgo exhibited a preference for manganese ions, similar to the previously studied cyanobacterial Argonautes LroAgo and SynAgo [16, 21]. This might be related to the physiology of cyanobacteria and increased content of manganese in cells, since it is a component of manganese cluster of the oxygen-evolving complex (OEC) of photosystem II (PSII) [49].

Measuring the temperature dependence and the rate of single-stranded DNA cleavage showed that CstAgo was active within the temperature range from 10 to 50°C, while the activity of CspAgo was observed only at 20°C and above. Moreover, CstAgo cleaved targets an order of magnitude faster than CspAgo. Hence, CstAgo has the highest activity at low temperatures among the known pAgos. Unfortunately, the removal of this protein from the original database makes it impossible to unambiguously determine its host species. Nevertheless, it can be assumed that the high activity of the studied proteins at low temperatures is associated with the adaptation of cyanobacteria to low environmental temperatures.

The data obtained suggest that the studied Argonautes can be potential tools for DNA manipulation, as they retained activity in a wide temperature range in the presence of standard concentrations of divalent cations, and did not depend on the 5′-terminal nucleotide of the guide (with the exception of reduced activity of CspAgo in the presence of cytosine at the 5′-end). Although CspAgo demonstrated lower activity toward single-stranded DNA, it specifically cleaved double-stranded plasmid DNA at physiological temperature.

The cleavage of double-stranded DNA by Argonautes requires the use of two guides complementary to the two DNA strands at the target locus, as each protein introduces a single-strand break. It was previously shown that some Argonautes from thermophilic and mesophilic bacteria can specifically cleave double-stranded DNA at high temperatures and excise target DNA fragments from plasmids [16, 23]. Thus, a technology based on the thermophilic Argonaute TtAgo has been created, which allows manipulations of plasmid DNA *in vitro* [39]. Several Argonautes can cleave plasmid DNA at 37°C, but their cleavage efficiency is low and strongly depends on the GC content of the cleavage site [47, 48]. The efficiency of double-stranded DNA cleavage can be increased by heating the reaction mixture [23, 47] or adding UvrD helicase, RecBC (which lacks nuclease activity but unwinds DNA), and SSB proteins [50, 51]. An alternative approach to the targeted cleavage of plasmids/double-stranded DNA *in vitro* using Argonautes is based on the application of catalytically inactive proteins directed to specific loci using guides and fused with FokI nuclease. To facilitate the binding of Argonautes to the target DNA, this technology uses modified oligonucleotides (peptide nucleic acids, PNA) complementary to both strands of the target and stabilizing the melted DNA region. This allowed a pair of chimeric proteins bound to the adjacent sites to form an active FokI dimer and introduce a double-strand break

even at medium and low temperatures, albeit with low efficiency [52].

In summary, the existing approaches involving Argonautes do not yet allow an efficient cleavage of target double-stranded DNA at physiological temperatures in the absence of additional factors. Significant activity of CstAgo and CspAgo proteins at moderate temperatures, as well as the ability of CspAgo to cleave double-stranded DNA at 37°C (albeit with low efficiency), makes these proteins promising candidates for further development of genome editors. The next steps in this direction may be optimization of properties of the studied proteins using artificial intelligence tools (as has been recently done for KmAgo [53]), characterization of new Argonautes among hyperpsychrophiles, and search for auxiliary factors capable of stimulating the cleavage of double-stranded DNA by Argonautes.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1134/S0006297925602680.

Contributions

D.M.G. and A.V.K. developed the concept and supervised the study; Yu.S.Z., E.V.K., and D.M.G. performed the experiments and collected the data; E.V.K., D.M.G., and A.V.K. analyzed the data and prepared the manuscript. All authors agree with the publication of the final version of the manuscript.

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Ethics approval and consent to participate

This work does not contain any studies involving human and animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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