Properties of the *Ureaplasma parvum* SMC protein related to its interaction with DNA

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Abstract

SMC (Structural Maintenance of Chromosomes) ATPase proteins are a component of complexes of the same name that participate in the spatial organization of DNA in all living organisms - in bacteria, archaea and eukaryotes. In bacteria, SMC complexes perform several functions, among which the function related to DNA condensation and segregation is the most studied. SMC complexes in bacteria are thought to extrude loops and thus compact DNA, as well as promote the separation of daughter nucleoids. In this work, we determined some properties of the SMC ATPase of *Ureaplasma parvum*, a member of the Mollicutes class with a reduced genome. The results indicate that the properties of this protein differ from those of the well-studied ortholog from *Bacillus subtilis*, in particular, *U. parvum* SMC binds to double-stranded DNA rather than single-stranded DNA. This protein has also been shown to compact DNA under *in vitro* conditions. The ATPase activity of *U. parvum* SMC was estimated and shown to be induced by DNA binding to SMC. The results of current paper indicate that *U. parvum* SMC cross-links DNA even in the absence of ATP.

Keywords: SMC, DNA segregation, spatial organization of DNA, *Ureaplasma parvum*

Introduction

SMC family protein complexes are widely distributed among species ranging from bacteria to eukaryotes and perform key functions in maintaining the spatial organization of DNA [1, 2]. SMC complexes consist of 3 main types of proteins: a dimer of the SMC ATPase proteins, a kleisin, and one of the auxiliary proteins like KITE or HAWK [3]. SMC proteins form a dimer (heterodimer in eukaryotes and homodimer in prokaryotes), which has a characteristic V-shape, with the arms of the SMC dimer formed by coiled-coils of about 50 nm in length. SMC proteins have ATPase activity stimulated by binding of the protein to DNA. Kleisin binds to the arms of the SMC dimer, forming a closed structure. KITE and HAWK proteins bind to SMC and kleisin and play an auxiliary and regulatory role. The most important

property of SMC complexes is loop extrusion - their ability to form loops from DNA molecules, and this ability is active, therefore these complexes are considered as one of the varieties of DNA translocases [1]. At present, loop extrusion has been demonstrated directly (under *in vitro* conditions at the single molecule level) for eukaryotic SMC complexes: condensins [4], cohesins [5] and Smc5/Smc6 complexes [6].

Bacteria have several types of SMC complexes, in particular, SMC-ScpAB [7], MukBEF [8], Wadjet [9] and others [10], among which SMC-ScpAB of *Bacillus subtilis* and MukBEF of *Escherichia coli* are the most studied. In these complexes, SMC and MukB are ATPases, ScpA and MukF are kleisins, and ScpB and MukE are auxiliary proteins of the KITE type [3]. The SMC-ScpAB and MukBEF complexes are involved in bacterial DNA segregation, and these complexes are involved in this process in different ways. The SMC-ScpAB complex of *B. subtilis* is loaded onto the bacterial chromosome with the participation of ParB and specifically binds to DNA at *parS* sites [11]. In contrast, the MukBEF complex of *E. coli* binds to the chromosome nonspecifically [12]. Another difference is that the action of SMC-ScpAB leads to the formation of a crescent-shaped nucleoid, whereas in bacteria with the MukBEF complex, a ring nucleoid is formed. Interestingly, the ability to extrude loops under *in vitro* conditions has not yet been proven for bacterial SMC complexes, although the results obtained *in vivo* [13], and some results of single-molecule experiments under *in vitro* conditions [14, 15] indicate the presence of such an ability.

SMC complexes are probably vital for DNA segregation in at least some bacteria under certain conditions. For example, the MukBEF complex of *E. coli* is vital under conditions of rapid growth [15]. Also, the high importance of SMC complexes is indicated by the fact that they are present in reduced Mollicutes genomes; even in the synthetic "minimal bacterium," genes encoding proteins of the SMC- ScpAB complex were conserved after directed deletion of approximately half of the genes [16]. It is of interest to elucidate the properties of SMC complexes in Mollicutes, since these complexes may be simpler organized than in bacteria with a "complete" genome, including simpler regulation of these complexes. Also, the functions of Mollicutes SMC complexes appear to overlap less with those of other protein complexes involved in DNA segregation than in more complex bacteria. In this work, the SMC protein (ATPase) from the SMC-ScpAB complex of *U. parvum* was investigated. The results indicate several important differences between this protein and the corresponding homolog of *B. subtilis*.

Materials and Methods

Creation of a genetic construct for the production of *U. parvum* SMC protein. Synthesis of the gene encoding the SMC protein of *U. parvum* with the codon composition optimized for *E. coli* was performed at IDT DNA. The nucleotide sequence of the gene was modified using the online resource Codon Optimization Tool (IDT DNA) to optimize the codon composition for efficient expression in *E. coli*. The synthesized gene was incorporated into the pJET1.2 plasmid for verification by Sanger sequencing. The resulting plasmid was transformed with *E. coli* XL-1 Blue strain. From the obtained plasmid based on the pJET1.2 vector, the gene of interest with the confirmed sequence was amplified using specific primers with NdeI and XhoI restriction sites added and incorporated into the pET21a plasmid using the standard restriction and ligation method. Synthesis of primers was performed by Evrogen. The resulting plasmids based on the pET21a vector were also verified by sequencing.

Production and isolation of *U. parvum* SMC protein. *E. coli* BL21 strain was used for the production of *U. parvum* SMC protein. Protein synthesis was confirmed by the presence of the corresponding band on the gel after electrophoresis (SDS-PAGE). The protein in this band was identified by mass spectrometry (peptide fingerprinting method performed as described in paper [17]).

Recombinant SMC protein was produced in a volume of 1 liter. Bacteria were cultured in a thermoshaker at 37°C until OD600=1. Expression of the gene encoding the recombinant protein was induced by adding IPTG (1 mM final concentration) to the culture; induction was performed in a thermoshaker at 22°C overnight. Cells were precipitated by centrifugation, the supernatant was removed and cells were resuspended in buffer A (50 mM Tris-HCl, 300 mM NaCl, 10 mM $MgCl_2$ and 1 mM PMSF, pH=8.0). Centrifugation was again performed at 3600 g, 4°C for 30 min and the precipitate was frozen at -20 °C.

The frozen precipitate was thawed on ice and resuspended in buffer A. Cells were disrupted on ice using a sonicator, with prior addition of lysozyme (1 mg/mL). The resulting lysate was centrifuged at 12000 g for 20 min at 4 °C. The supernatant was collected and filtered. The resulting lysate was applied to a 1 mL HisCap Smart 6FF column (Smart-Lifesciences) using an Akta Purifier 10 chromatograph (GE Healthcare) and was washed with buffer B (buffer A + 500 mM imidazole), increasing its concentration in steps. Samples of 1 mL were taken from each peak of the chromatogram and frozen in liquid nitrogen and then stored at -80 °C. The obtained samples were analyzed by SDS-PAGE electrophoresis. SMC protein concentration was estimated by absorbance (OD280) with correction for molecular weight (MW=112 kDa) and molar extinction coefficient (ε=88443 $cm^{-1}*M^{-1}$) of *U. parvum* SMC protein.

Electrophoretic mobility shift assay of DNA in the presence of SMC protein in a polyacrylamide gel. Two fluorescently labeled DNA molecules were used to analyze the shift of electrophoretic mobility of DNA (EMSA) in a polyacrylamide gel: a 110 bp fragment of the human GRIN2B gene and a 110 bp sequence from the genome of *U.* parvum (GC content of about 20%), which was designated MTA (abbreviation for the gene encoding the 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase protein). Fluorescently labeled molecules were obtained by PCR using primers, one of which had a Cy3 label at the 5'-end. To obtain partially single-stranded DNA molecules, melting of double-stranded DNA at 95 °C and rapid cooling on ice was performed.

Reaction mixtures (20 μL) contained 20 mM Tris-HCl, pH 7.5, 7.5 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT and SMC protein (final concentration 0.065 to 1.04 μM). The mixtures were centrifuged at 16000 g for 15 min, the supernatants were withdrawn and incubated at 37 °C for 5 min. Fluorescently labeled DNA (partially single-stranded or fully double-stranded molecules) was then added to each sample to a final concentration of 5 nM. The ratio of DNA to protein molecules was 1:10, 1:20, 1:20, 1:50, 1:100, 1:200, respectively.

After incubation in the dark at 37 °C for 30 min, glycerol was added to the mixture up to 10%, and samples were analyzed by native electrophoresis in a 5% polyacrylamide gel in TBE buffer at 70 V for 50 min on ice. The gel was pre-equilibrated in the same buffer at 80 V for 30 min. The gel was visualized using the ChemiDoc gel documentation system (Bio-Rad) with Cy3 dye channel selection.

Electrophoretic mobility shift assay of DNA in the presence of SMC protein in agarose gel. The DNA substrates used were circular or linearized plasmid pUC19 dual (9396 bp) [18]. The following protocol was used for most subsequent experiments. The SMC protein was centrifuged at 21130 g for 10 min at 4 °C to get rid of insoluble protein, after which the supernatant was collected. Next, 4 μg of SMC protein were added to the reaction mixture based on one of the buffers (see below), followed by the addition of 200 ng of circular or linear pUC19 dual plasmid (DNA:protein ratio of 1:1000). In addition, two control samples were prepared: one of them did not contain SMC protein but had the same amount of DNA as the rest of the reaction mixtures, and the second one did not contain DNA but had SMC protein present at the concentration as in the tested samples. At the very end, ATP was added to the samples. Next, the samples were incubated for 40 minutes at 37° C. Samples were loaded on a 0.7% agarose gel based on Tris-acetate buffer. Electrophoresis was performed at a constant voltage of 80 V for 25 minutes. After electrophoresis, the gel was incubated in TAE containing ethidium bromide (1/10000) to visualize DNA.

Several buffers of different compositions were used for different experiments. To elucidate the effect of ATP on the DNA-binding properties of the SMC protein, identical reaction mixtures were prepared based on buffer with pH = 7 (20 mM Tris-HCl, 1 mM DTT, 7.5 mM KCl, 2.5 mM $MgCl_2$, pH = 7) that differed in ATP concentration: 0 mM, 2.5 mM, 5 mM, 7.5 mM, 10 mM.

Similarly, to elucidate the effect of pH of buffer solutions on the DNA-binding properties of SMC protein, reaction mixtures were prepared based on buffers with different pH values (pH = 6, 7, 8, 9, 10). For pH = 7, 8, 9, 10, buffers based on 20 mM Tris-HCl were used, for pH = 6 - buffer based on 20 mM MES. All mixtures contained 1 mM DTT, 7.5 mM KCl, 2.5 mM $MgCl_2$, and 5 mM ATP.

To evaluate the effect of KCl concentration on the DNA-binding properties of SMC protein, we used mixtures based on pH = 6 buffer (20 mM MES, 1 mM DTT, 2.5 mM $MgCl_2$, 5 mM ATP, pH = 6) with different concentrations of KCl salt: 0 mM, 25 mM, 50 mM, 75 mM, 100 mM.

To evaluate the effect of $_{MgCl2}$ concentration on the DNA-binding properties of SMC protein, we used buffer-based mixtures at pH = 6 (20 mM MES, 1 mM DTT, 7.5 mM KCl, 5 mM ATP, pH = 6) with different concentrations of $MgCl_2$: 0 mM, 10 mM, 50 mM, 100 mM, 200 mM.

Additionally, the effect of DNA:SMC ratio on the interaction of SMC with DNA was determined. Reaction mixtures based on pH 6 buffer (20 mM MES, 1 mM DTT, 7.5 mM KCl, 2.5 mM $_{MgCl2}$, 5 mM ATP, pH = 6) were prepared, varying among themselves the ratio of DNA molecules: SMC: 1:1000, 1:800, 1:600, 1:400, 1:200 (varying the amount of protein, with a constant amount of DNA - 200 ng).

Measurement of ATPase activity. The reaction mixture, which contained 20 mM MES, pH 6, 7.5 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 656 nM SMC protein (1.47 μg), 1.64 nM DNA (ring plasmid pUC19dual - 200 ng), and 1 mM ATP, was incubated at 37 °C for a specific time interval (5, 10, 15, 20 min). 20 μl of the reaction mixture was transferred into a 96-well flat-bottom plate. 200 μl of color reagent (1 part 4.2% ammonium molybdate, 3 parts 0.045% malachite green solution, 1/50 part 2% Nonidet P-40 solution) was added to the samples and mixed. After 1 min, 20 μl of 34% sodium citrate solution was added and stirred. The absorbance of the solutions at 620 nm wavelength was analyzed using a platereader spectrophotometer. To determine the concentration of free phosphate in the samples, a calibration line corresponding to a standard with known values of free phosphate

concentration was used. The standard used was 1 mM NaH_2PO_4, which was diluted with water to concentrations: 0, 50, 100, 150, 200 μM.

Molecular combing. λ phage DNA, 48502 bp in length, pre-stained with YOYO-1 fluorescent dye was used for molecular combing. The reaction mixture contained 20 mM Tris-HCl, pH = 7.5, 7.5 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1.3 μM SMC and 0.3 nM λ DNA. Reaction mixtures to which no ATP or SMC protein was added were used as controls. The reaction mixtures were incubated in a thermostat at 37 °C for 30 min. Then, 100 mM sodium acetate buffer (pH = 5.5) was added to each mixture for subsequent application of DNA to the coverslip by molecular combing method [19].

Pre-cleaned (by plasma cleaning in the PlasmaCleaner) 22x22 mm coverslips were hydrophobically coated by evenly applying 200 μl of polystyrene solution in benzene using a spin-coater, then the coverslips were washed with water. The polystyrene-coated coverglass was incubated in buffer with the reaction mixture for 5 min and then the glass was slowly pulled through a meniscus of solution to unfold and anchor the DNA molecule on the glass. A standard fluorescence microscopy method, the YFP fluorescence channel, was used to visualize and capture the dye-labeled DNA.

DNA manipulation by laser tweezers. Optical trapping experiments were performed to analyze SMC protein binding to individual double-stranded and single-stranded DNA molecules. DNA manipulation was carried out inside a five-channel microfluidic system (uFlux, Lumicks), using two polystyrene microspheres coated with streptavidin (Spherotech, 2.1 μm diameter) and held by two optical traps according to the method presented in [20, 21]. A 11070 bp long linear DNA molecule (prlC) was used as substrate. The solution contained Tris-HCl buffer (pH=7.5, 25 mM), 7.5 mM KCl, 2.5 mM $MgCl_2$, 0.02% BSA.

Results and Discussion

SMC *U. parvum* forms complexes with double-stranded DNA. Analysis of the shift in electrophoretic mobility of DNA in the presence of SMC protein in polyacrylamide and agarose gels showed that the SMC protein of *U. parvum* is able to bind to DNA (see Figure 1). The results indicate that this protein binds to double-stranded DNA, while the presence of single-stranded sites on the DNA molecule (obtained by rapid annealing of the PCR product) does not stimulate the interaction of SMC with DNA. The binding of SMC of *U. parvum* to double-stranded DNA is an interesting observation, since in the paper [22] using a similar method it was shown that the SMC homologue from *B. subtilis* binds to single-stranded DNA and virtually does not bind to double-stranded DNA.

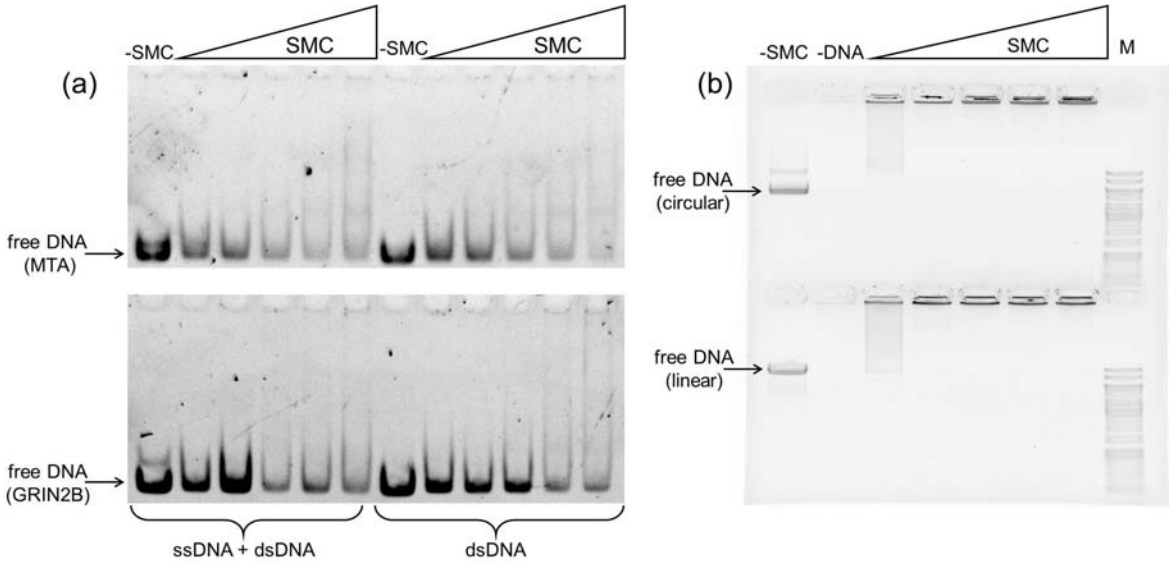

Figure 1. Effect of SMC *U. parvum* on the electrophoretic mobility of DNA. (a) – binding of *U. parvum* SMC protein to linear DNA fragments visualized in polyacrylamide gel (EMSA). The binding of SMC to a mixture of single- and double-stranded DNA obtained by rapid annealing of the PCR product (ssDNA + dsDNA) and to double-stranded DNA (dsDNA) is shown. Top – binding of *U. parvum* SMC protein to 110 bp *MTA* fragment of the *U. parvum* genome. Bottom – binding of *U. parvum* SMC protein to a 110 bp fragment of the human *GRIN2B* gene. Samples with a stepwise increase in the amount of SMC from 0 (-SMC) to the maximum amount according to the ratios of DNA:SMC molecules (1:10, 1:20, 1:50, 1:100, 1:200, respectively) are shown. (b) – binding of *U. parvum* SMC protein to the circular and linear form of DNA (9396 bp plasmid pUC19-dual) visualized in agarose gel (EMSA). Top – binding of SMC protein to the ring form of the plasmid. Bottom – binding of the SMC protein to the linear form of the plasmid. Samples with a stepwise increase in SMC amount from 0 (-SMC) to the maximum amount according to the ratios of DNA:SMC molecules (1:200, 1:400, 1:600, 1:800, 1:1000, respectively) are shown. M – marker (Thermo Scientific GeneRuler 1kb DNA Ladder), -DNA – control sample without DNA addition. -SMC – control sample without SMC addition

It is known that *B. subtilis* SMC is loaded onto the chromosome by ParB at *parS* sites, which ensures specific binding of SMC protein, as well as the SMC complex as a whole, to the chromosome in the origin of replication [11]. In contrast, the SMC-like MukBEF complex of *E. coli* binds well to double-stranded DNA, and the binding is nonspecific [23], although in

general the role of MukBEF and SMC-ScpAB complexes is practically the same. It can be assumed that if the SMC of *B. subtilis* binds to DNA without the participation of ParB, the binding would be nonspecific, since it is ParB that specifically binds to *parS* sites. The data that SMC of *U. parvum* interacts with double-stranded DNA without additional factors may indirectly indicate that this protein binds nonspecifically to the *U. parvum* chromosome. Indeed, our results generally support the assumption that *U. parvum* SMC binds to DNA nonspecifically, since the shift was observed almost as a continuous trace (smear) on the track, as well as in the form of complexes stuck in the wells of the gel (obviously, because of formation of sufficiently large SMC + DNA complexes). It is worth noting that the binding of SMC to DNA in the above experiments occurred without the addition of ATP. The nonspecific binding of SMC and DNA is also indirectly confirmed by our data that SMC of *U. parvum* fused with the fluorescent protein mMaple2 shows co-localization with DNA without pronounced foci relative to the nucleoid during heterologous expression of the corresponding gene in *Mycoplasma gallisepticum* cells (data not presented).

The *U. parvum* SMC protein was also shown to interact approximately equally with the circular and linear forms of DNA (see Figure 1b). This result is unexpected, as other SMC proteins have been shown to preferentially bind to the circular form of DNA. It is well known that various SMC complexes (including condensins, cohesins, Smc5-Smc6, etc.) show topological binding to DNA as it depends on the topology of the DNA molecule: binding to circular DNA is usually more pronounced than binding to linear DNA [24-26]. For example, for the RecN protein, which is an SMC-like protein, a much stronger interaction of RecN with circular DNA (especially single-stranded DNA) than with linear DNA was shown under conditions similar to those of this experiment [27]. We were unable to find published results on the properties of the *B. subtilis* SMC protein under the conditions of experiments similar to those performed in this work, so such a comparison cannot be made directly. The fact that SMC of *U. parvum* binds to DNA independently of its topology requires further investigation. One of the possible explanations for the identical binding of the SMC *U. parvum* protein to circular and linear DNA is the formation of unusually stable SMC/DNA complexes that do not dissociate under the conditions of the experiments performed. The high stability of such complexes may be related, among other things, to the relatively low ATPase activity of SMC *U. parvum* (see below).

The interaction of *U. parvum* SMC with DNA is enhanced by the addition of ATP. Analysis of the shift in electrophoretic mobility of different forms of double-stranded DNA

(circular supercoiled and linear) in the presence of SMC protein in an agarose gel showed similar results: SMC interacts with DNA and forms complexes with it even in the absence of ATP in the solution. It was possible to demonstrate the dependence of the DNA-binding properties of the investigated protein on the ATP concentration (see Figure 2a). Increasing the ATP concentration enhanced the interaction of SMC protein with DNA: more active formation of protein/DNA complexes in the gel well was observed with increasing ATP concentration (see Figure 2a). At high ATP concentrations (more than 5 mM), most of the 9396 bp molecules of plasmid pUC19 dual appeared bound to SMC. Since at ATP concentration of 5 mM most of the DNA was complexed in SMC, this concentration was used in the following experiments.

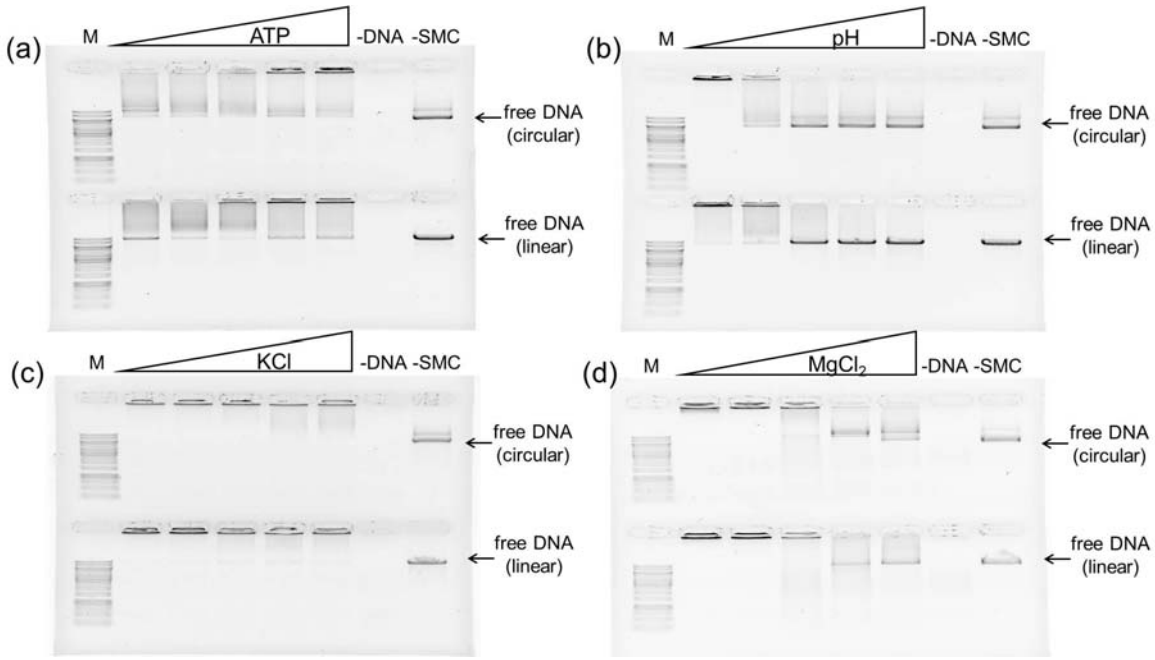

Figure 2: Effect of medium composition on the binding of *U. parvum* SMC protein to DNA (9396 bp plasmid pUC19-dual) visualized in agarose gel by EMSA method. (a) – Effect of ATP concentration. ATP concentrations were 0, 2.5, 5, 7.5 and 10 mM, respectively. (b) – Effect of solution pH. The pH values were 6, 7, 8, 9 and 10 respectively. (c) – Effect of KCl concentration. The concentrations of KCl were 0, 25, 50, 75 and 100 mM, respectively. (d) – Effect of $MgCl_2$ concentration. The concentrations of $MgCl_2$ were 0, 10, 50, 100 and 200 mM, respectively. In all parts of the figure, the binding of SMC protein to the circular plasmid is shown at the top, while the binding to the linear plasmid is shown at the bottom. M – marker (Thermo Scientific GeneRuler 1kb DNA Ladder), -DNA – control sample without DNA addition. -SMC – control sample without SMC addition

This observation indicates another difference between SMC of *U. parvum* and SMC of *B. subtilis*: for the latter protein it has been shown that there is no effect of ATP on protein-DNA binding [28]. It is interesting to note that in the same paper, a mutant form of *B. subtilis* SMC (E1118Q) with reduced ATPase activity, in contrast to the wild-type protein, was shown to stimulate the interaction of SMC with DNA upon addition of ATP. It can be hypothesized that the *U. parvum* SMC protein, which has weaker ATPase activity (see below), is similar to the mutant SMC of *B. subtilis* in this sense, as its interaction is enhanced in the presence of ATP. Another SMC-like protein from *E. coli*, MukB, enhances interaction with DNA in the presence of ATP [29]. It is interesting to note that eukaryotic cohesin also exhibits ATP dependence for binding to DNA [30].

The interaction of *U. parvum*SMC with DNA was weakened with increasing pH and concentration of KCl, $MgCl_2$. Increasing pH above 7 weakened the interaction of SMC protein with DNA: less active formation of SMC/DNA complexes in the gel well was observed at higher pH (see Figure 2b). Since pH = 6 was found to be optimal for protein-DNA binding, it was the pH = 6 buffer that was used in subsequent experiments. It was found that increasing the concentration of KCl starting at 50 mM inhibited the interaction between DNA and SMC (see Figure 2c). It was also found that increasing the concentration of $MgCl_2$ starting at 50 mM inhibited the interaction between DNA and SMC (see Figure 5d). We were unable to find comparative data in the literature on the effect of pH and monovalent and divalent ion concentrations on the interaction of SMC orthologs with DNA, although for some homologs it was shown that salt inhibits the intaraction. Nevertheless, the data obtained here are important for understanding the functional activity of this protein in living *U. parvum* cells. Taking into account physiological values of these parameters in bacterial cells (pH about 7, about 100 mM KCl and 10 mM $MgCl_2$, it can be assumed that SMC/DNA complexes should be formed in *U. parvum* cells.

SMC *U. parvum* exhibits ATPase activity stimulated by DNA. Spectrophotometric analysis of the ATPase activity of the *U. parvum* SMC protein using malachite green assay confirmed the assumption that this protein hydrolyzes ATP in the presence of DNA. Based on a linear approximation of the relationship corresponding to standards with known values of free phosphate concentration, the rate of ATP hydrolysis by the SMC *U. parvum* protein was calculated. It was determined that one molecule of SMC protein (monomer) hydrolyzes 3.8 ± 1.1 (mean ± standard deviation) ATP molecules per minute. In addition, experiments

showed that the SMC protein of *U. parvum* is also able to hydrolyze ATP in the absence of DNA, but the rate of hydrolysis is about 2 times lower. These calculations were made assuming that hydrolysis of one molecule of ATP releases one molecule of free phosphate. Thus, as previously for the SMC protein of *B. subtilis*, we were able to demonstrate DNA-stimulated ATPase activity in this work. At the same time, the ATPase activity of SMC *U. parvum* is about an order of magnitude lower than that of SMC *B.* subtilis (about 30 molecules per minute) [28] and comparable to that of MukB. [23].

The interaction of *U. parvum* SMC with DNA prevents the latter from straightening by molecular combing. Using the molecular combing method, it was demonstrated that the *U. parvum* SMC protein binds to DNA, preventing it from straightening on the coverslip under the influence of the surface tension force acting on DNA in the liquid-air phase transition zone. It can be seen that DNA straightening is not observed both in the absence of ATP and in the presence of 1 mM ATP (see Figure 3). This result is consistent with the results obtained by electrophoretic mobility shift assay, as it confirms the binding of SMC *U. parvum* c DNA. In addition, the molecular combing method allows us to conclude that this protein compacts one or more DNA molecules, apparently by forming loops from one molecule or by cross-linking several DNA molecules simultaneously.

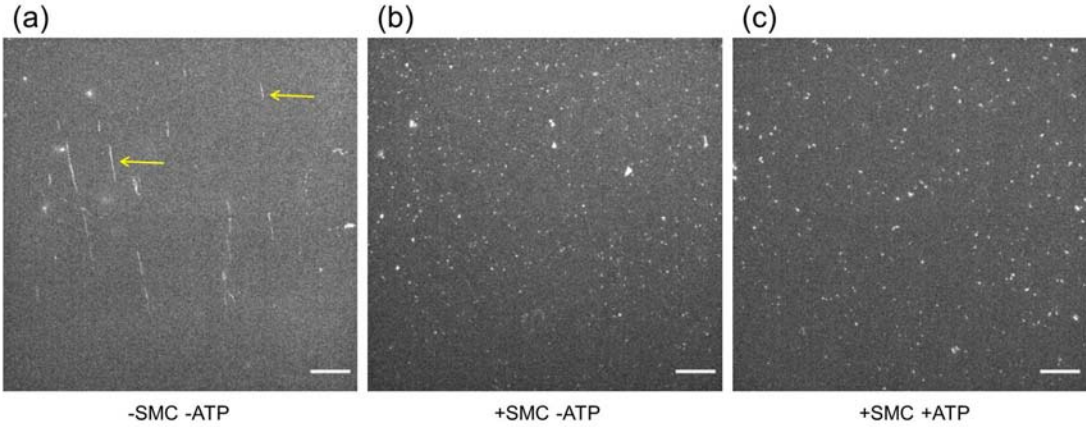

Figure 3: Visualization of λ bacteriophage DNA stretched by molecular combing. DNA was stained with fluorescent dye YOYO-1 by fluorescence microscopy. (a) – λ DNA incubated without SMC of *U. parvum*. Arrows indicate straightened DNA molecules. (b) – λ DNA incubated with *U. parvum* SMC without ATP. (c) – λ DNA incubated with *U . parvum* SMC and with 1 mM ATP. The scale size is 15 μm

SMC *U. parvum* shortens DNA molecules under single-molecule experimental conditions. Using laser tweezers, it was demonstrated that the *U. parvum* SMC protein binds to double-stranded DNA molecules attached at the ends to microspheres, as incubation of a relaxed (unstretched) DNA molecule with SMC without the addition of ATP leads to subsequent DNA shortening at the same tension force compared to the original DNA molecule (see Figure 4). It is interesting to note that incubation of the stretched DNA molecule with SMC solution does not result in a similar effect. Based on this observation, we can make an assumption that in the case of relaxed DNA molecule, the SMC is able to form spatial complexes with it (possibly, to form loops). Probably, SMC forms cross-links between different parts of DNA, which leads to shortening of the latter. This observation is consistent with other results of this work and indicates high stability of SMC/DNA complexes. It is interesting to note that DNA shortening occurred in the absence of ATP, which is similar to the result obtained by molecular combing (see Figure 3).

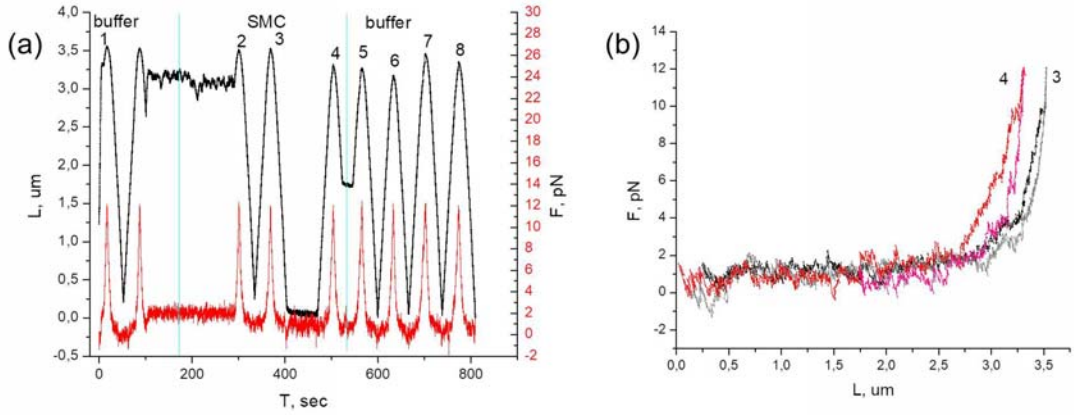

Figure 4: *U. parvum* SMC interaction with DNA under single-molecule experiment conditions visualized by laser tweezers. A linear DNA molecule with a length of 11070 bp (prlC) was used as substrate. The solution contained Tris-HCl buffer (pH=7.5, 25 mM), 7.5 mM KCl, 2.5 mM $MgCl_2$, 0.02%BSA. (a) – an example of a single-molecule experiment with a single DNA molecule that was subjected to stretching and relaxation upon incubation with SMC. The time dependence of the molecule length (black curve, scale on the left) and its tension force (red curve, scale on the right) are shown. Blue vertical lines delimit the time intervals in which the DNA molecule was placed in a solution without SMC (buffer) and in a solution with SMC protein (SMC). Numbers indicate successive acts of DNA elongation and relaxation. (b) – examples of the dependences of the tension force on the DNA length (force-extension curves) based on the dependences (a). Curve (3) corresponds to the act of

elongation (3) of the DNA molecule after its incubation in the stretched state in the presence of SMC in graph (a), curve (4) – respectively after incubation in the relaxed state. It can be seen that in the latter case the DNA molecule became shorter at the same tension force

In this work, we were unable to demonstrate active extrusion of loops by the SMC protein of *U. parvum* using laser tweezers and using another single-molecule method, namely the analysis of U-shaped DNA molecules in the liquid flow [6] (данные не представлены). The most likely reason for this is our use of only the SMC ATPase rather than the whole SMC-ScpAB complex. Probably, in the absence of kleisin (ScpA) and KITE (ScpB), SMC is able to bind to DNA and ATP, hydrolyze ATP; ATP binding and/or hydrolysis somehow stimulate the formation of DNA spatial structures (i.e. SMC cross-links between different segments of one or more DNA molecules), which follows from the data obtained by EMSA (see Figure 1a). Interestingly, the ability to compact genomic DNA independently of ATP binding and hydrolysis was recently demonstrated for the SMC protein of *B. subtilis* under *in vivo* conditions [31], ScpAB modulate this compactization. A similar property was shown for MukB protein - under *in vitro* conditions this protein compacted DNA in *the* absence of ATP [12]. Given the relatively low ATP hydrolysis rate by the *U. parvum* SMC protein, it is important to explore the connection between its ATPase activity and its ability to compact DNA in the future.

In this study, the ATPase activity of the SMC protein from *U. parvum* was investigated. Notably, the properties of this protein differ significantly from those of its ortholog, *B. subtilis* SMC. Specifically, *U. parvum* SMC interacts with double-stranded DNA (dsDNA) rather than single-stranded DNA (ssDNA), and it can bind both linear and circular DNA molecules. Unlike *B. subtilis* SMC, the interaction of *U. parvum* SMC with DNA is enhanced at higher ATP concentrations. The ATPase activity of *U. parvum* SMC was evaluated and found to be comparable to that of *E. coli* MukB but approximately an order of magnitude lower than that of *B. subtilis* SMC.

For the first time, the influence of KCl, $MgCl_2$, and pH on DNA interaction was determined for SMC protein. This revealed more effective DNA binding at low ion concentrations (less than 100 mM) and low pH (below 7). Additionally, DNA compaction was observed upon binding to *U. parvum* SMC using molecular combing and laser tweezers methods, independently of ATP presence. Further research using the complete SMC-ScpAB complex, as well as other proteins interacting with this complex, is necessary to demonstrate the active DNA loop extrusion capability of this complex.

Conclusions:

1. *U. parvum* SMC binds to double-stranded circular and linear DNA.
2. The interaction of *U. parvum* SMC with DNA is inhibited at high KCl, $MgCl_2$ concentrations (above 100 mM), as well as at high pH values (above 7).
3. The interaction of *U. parvum* SMC with DNA rises with increasing ATP concentration.
4. *U. parvum* SMC demonstrates DNA-dependent ATPase activity. One SMC monomer hydrolyzes 3.8 ATP molecules per minute.
5. *U. parvum* SMC shortens the DNA as follows from molecular combing and laser tweezers data.

Competing interests statement

The authors declare no competing interests.

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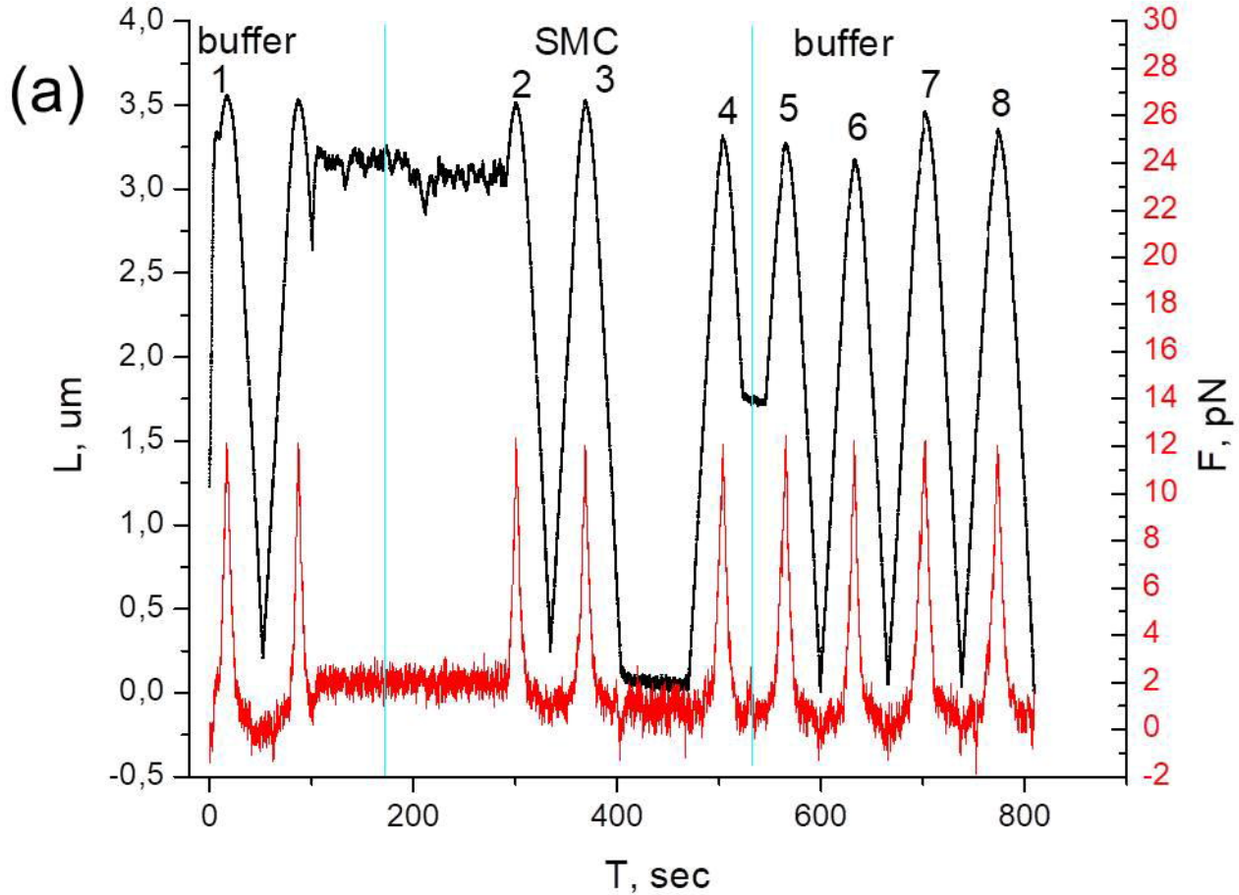
Author Contributions: R.N.: Investigation, Formal analysis, Writing; S.V.: Investigation, Writing; B.D.: Investigation, Formal analysis; A.A.: Investigation, Formal analysis; M.N.: Funding acquisition; V.A.: Supervision, Writing.

References

1. Kim, E., R. Barth, and C. Dekker, *Looping the Genome with SMC Complexes.* Annu Rev Biochem, 2023.
2. Yatskevich, S., J. Rhodes, and K. Nasmyth, *Organization of Chromosomal DNA by SMC Complexes.* Annu Rev Genet, 2019. **53**: p. 445-482.
3. Морозова, Н.Е., А.С. Потысьева, and А.Д. Ведяйкин, *Особенности структуры и функций бактериальных комплексов SMC.* Цитология, 2023. **65**(6): p. 1-13.

4. Ganji, M., et al., *Real-time imaging of DNA loop extrusion by condensin.* Science, 2018. **360**(6384): p. 102-105.
5. Davidson, I.F., et al., *DNA loop extrusion by human cohesin.* Science, 2019. **366**(6471): p. 1338-1345.
6. Pradhan, B., et al., *The Smc5/6 complex is a DNA loop-extruding motor.* Nature, 2023. **616**(7958): p. 843-848.
7. Minnen, A., et al., *Control of Smc Coiled Coil Architecture by the ATPase Heads Facilitates Targeting to Chromosomal ParB/parS and Release onto Flanking DNA.* Cell Rep, 2016. **14**(8): p. 2003-16.
8. Japaridze, A., et al., *MukBEF-dependent chromosomal organization in widened Escherichia coli.* Front Microbiol, 2023. **14**: p. 1107093.
9. Deep, A., et al., *The SMC-family Wadjet complex protects bacteria from plasmid transformation by recognition and cleavage of closed-circular DNA.* Mol Cell, 2022. **82**(21): p. 4145-4159.e7.
10. Nolivos, S. and D. Sherratt, *The bacterial chromosome: architecture and action of bacterial SMC and SMC-like complexes.* FEMS Microbiol Rev, 2014. **38**(3): p. 380-92.
11. Gruber, S. and J. Errington, *Recruitment of condensin to replication origin regions by ParB/SpoOJ promotes chromosome segregation in B. subtilis.* Cell, 2009. **137**(4): p. 685-96.
12. Zhou, M., *DNA sliding and loop formation by E. coli SMC complex: MukBEF*. Biochemistry and Biophysics Reports, 2022. **31**: p. 101297.
13. Mäkelä, J. and D. Sherratt, *SMC complexes organize the bacterial chromosome by lengthwise compaction.* Current Genetics, 2020. **66**(5): p. 895-899.
14. Pradhan, B., et al., *Loop extrusion-mediated plasmid DNA cleavage by the bacterial SMC Wadjet complex.* bioRxiv, 2024.
15. Zhou, M., *DNA sliding and loop formation by E. coli SMC complex: MukBEF*. Biochem Biophys Rep, 2022. **31**: p. 101297.
16. Hutchison, C.A., et al., *Design and synthesis of a minimal bacterial genome.* Science, 2016. **351**(6280): p. aad6253.
17. Lavysh, D., et al., *The genome of AR9, a giant transducing Bacillus phage encoding two multisubunit RNA polymerases.* Virology, 2016. **495**: p. 185-96.
18. Morozova, N., et al., *Temporal dynamics of methyltransferase and restriction endonuclease accumulation in individual cells after introducing a restriction-modification system.* Nucleic Acids Res, 2016. **44**(2): p. 790-800.
19. Bensimon, A., et al., *Alignment and sensitive detection of DNA by a moving interface.* Science, 1994. **265**(5181): p. 2096-8.
20. Alekseev, A., et al., *A new insight into RecA filament regulation by RecX from the analysis of conformation-specific interactions.* Elife, 2022. **11**.

21. Alekseev, A., et al., *Single-molecule characterization of compressed RecA nucleoprotein filaments.* Biochem Biophys Res Commun, 2022. **614**: p. 29-33.
22. Hirano, M. and T. Hirano, *ATP-dependent aggregation of single-stranded DNA by a bacterial SMC homodimer.* Embo j, 1998. **17**(23): p. 7139-48.
23. Hayama, P.D.R., et al., *The MukB-ParC Interaction Affects the Intramolecular, Not Intermolecular, Activities of Topoisomerase IV.* The Journal of biological chemistry, 2013. **288**.
24. Cuylen, S., J. Metz, and C.H. Haering, *Condensin structures chromosomal DNA through topological links.* Nat Struct Mol Biol, 2011. **18**(8): p. 894-901.
25. Haering, C.H., et al., *The cohesin ring concatenates sister DNA molecules.* Nature, 2008. **454**(7202): p. 297-301.
26. Kanno, T., D.G. Berta, and C. Sjögren, *The Smc5/6 Complex Is an ATP-Dependent Intermolecular DNA Linker.* Cell Rep, 2015. **12**(9): p. 1471-82.
27. Keyamura, K. and T. Hishida, *Topological DNA-binding of structural maintenance of chromosomes-like RecN promotes DNA double-strand break repair in Escherichia coli.* Communications Biology, 2019. **2**(1): p. 413.
28. Hirano, M. and T. Hirano, *Positive and negative regulation of SMC-DNA interactions by ATP and accessory proteins.* Embo j, 2004. **23**(13): p. 2664-73.
29. Chen, N., et al., *ATP-induced shrinkage of DNA with MukB protein and the MukBEF complex of Escherichia coli.* J Bacteriol, 2008. **190**(10): p. 3731-7.
30. Arumugam, P., et al., *ATP hydrolysis is required for cohesin's association with chromosomes.* Curr Biol, 2003. **13**(22): p. 1941-53.
31. Knust, T. and P.L. Graumann, *Bacillus subtilis Smc condenses chromosomes in a heterologous cell system, which is down-regulated by ScpAB.* BMC Research Notes, 2020. **13**(1): p. 524.


(a)
buffer
SMC
buffer
1
2
3
4
5
6
7
8
L, um
F, pN
T, sec
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3,5
3,0
2,5
2,0
1,5
1,0
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-0,5
0
200
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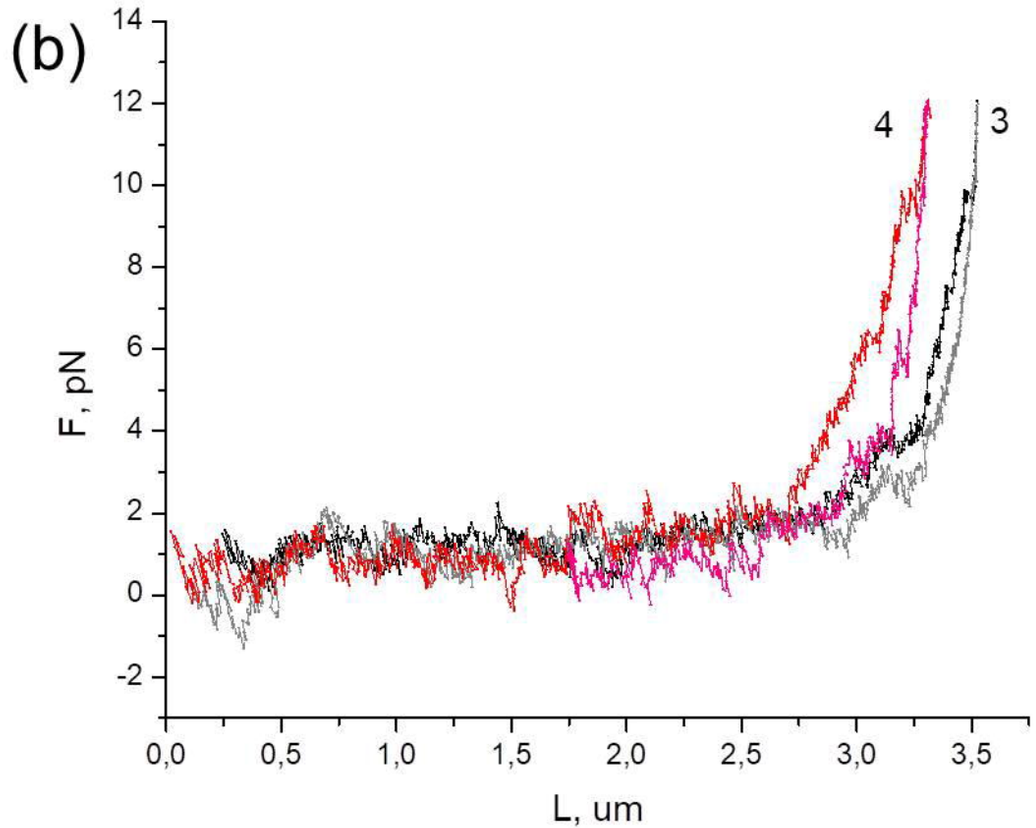
(b)
4
3
F, pN
L, um
14
12
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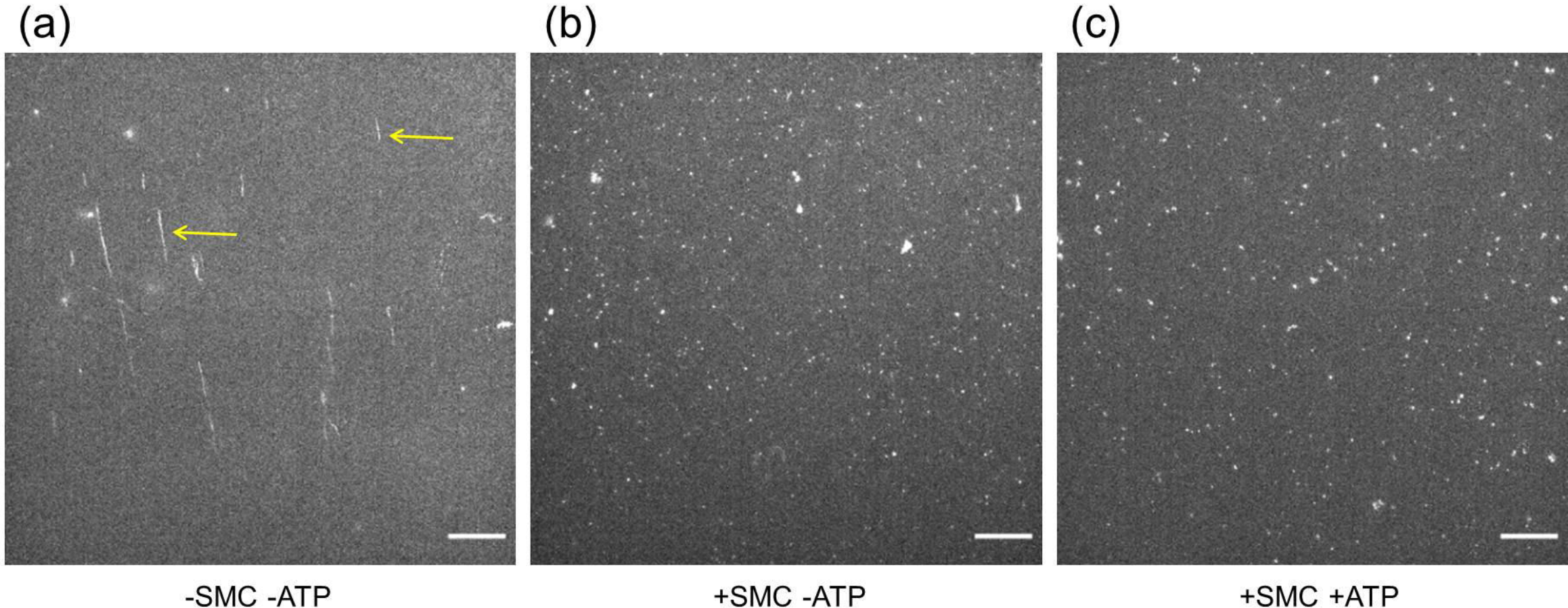
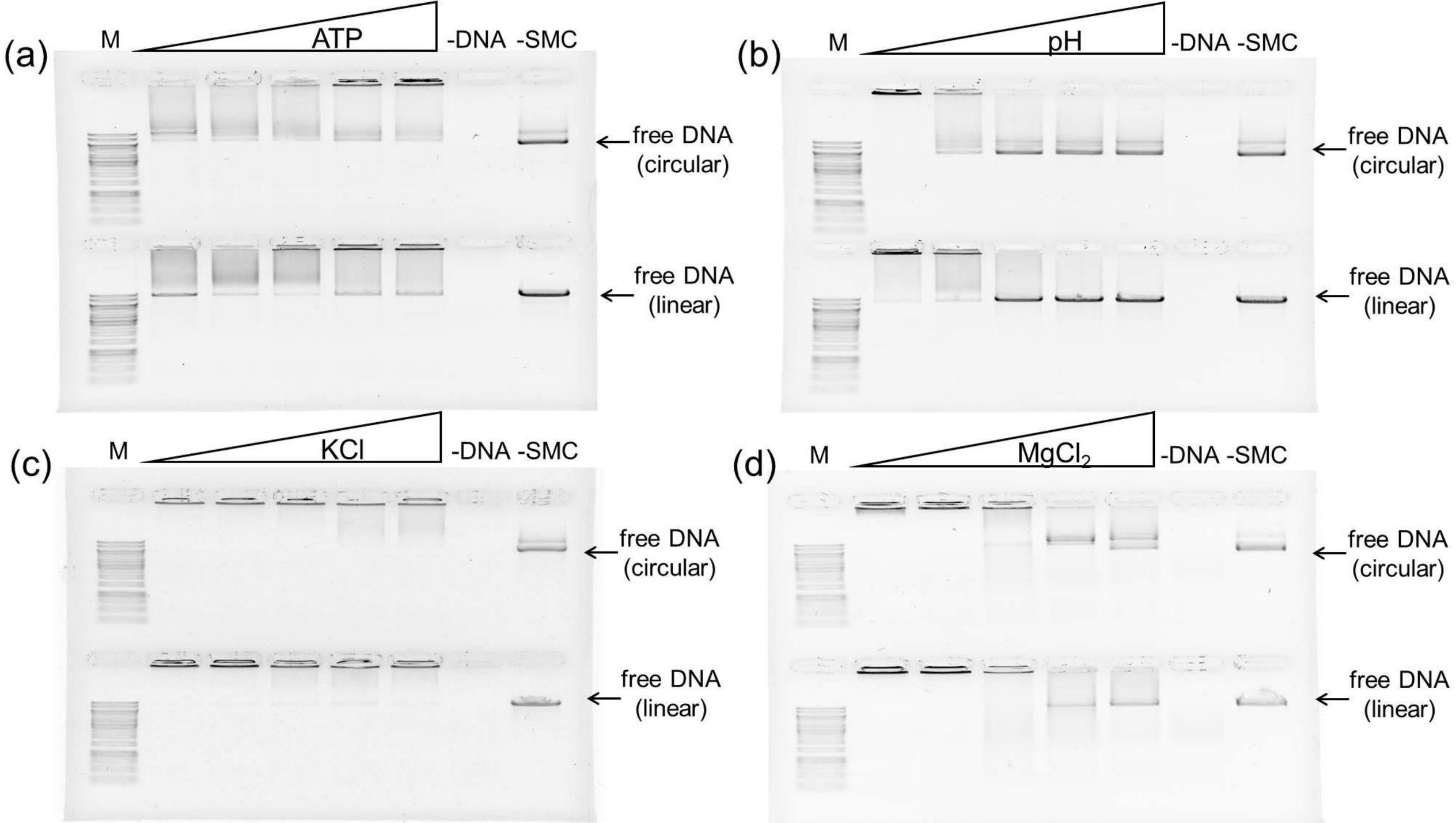
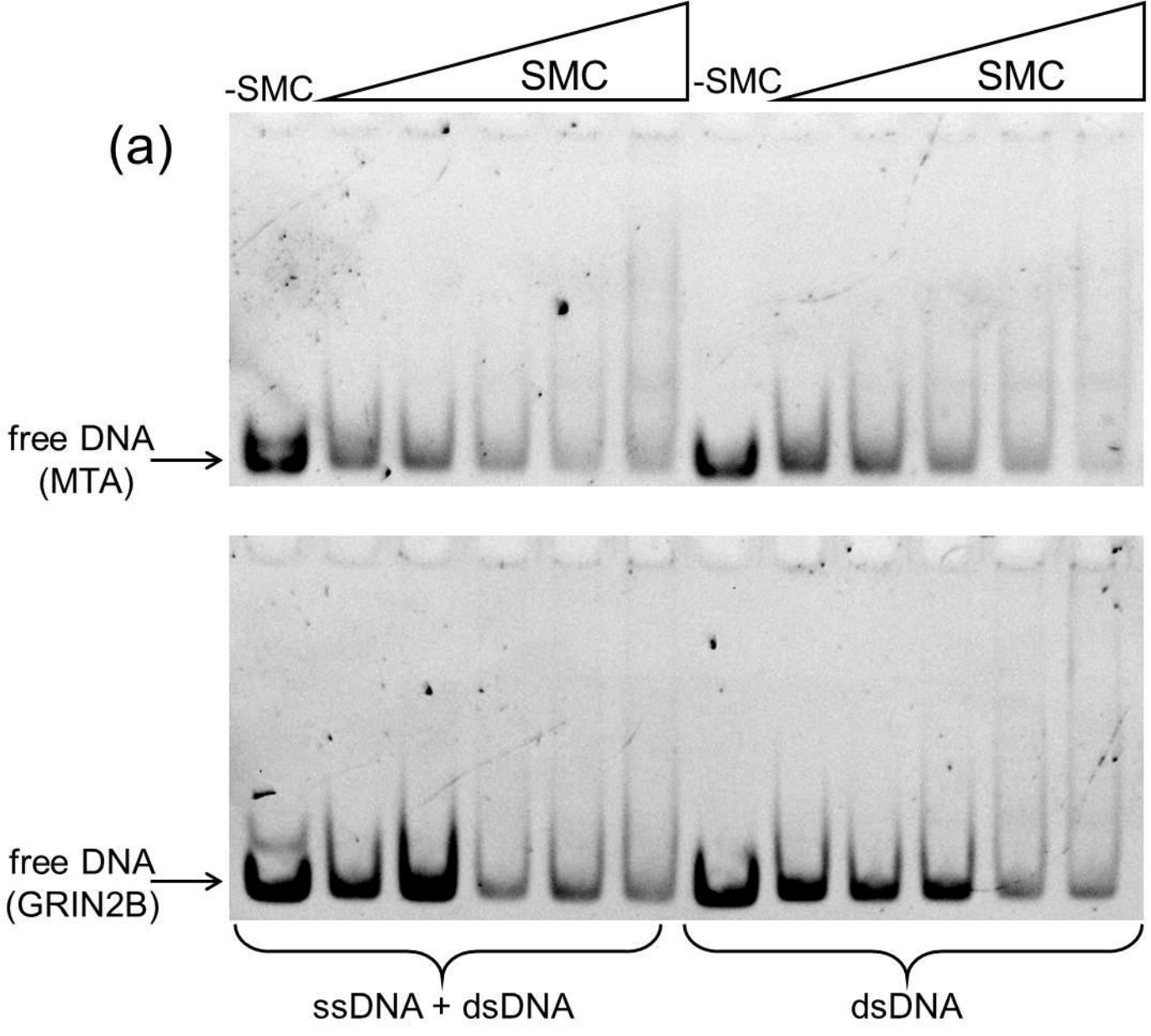
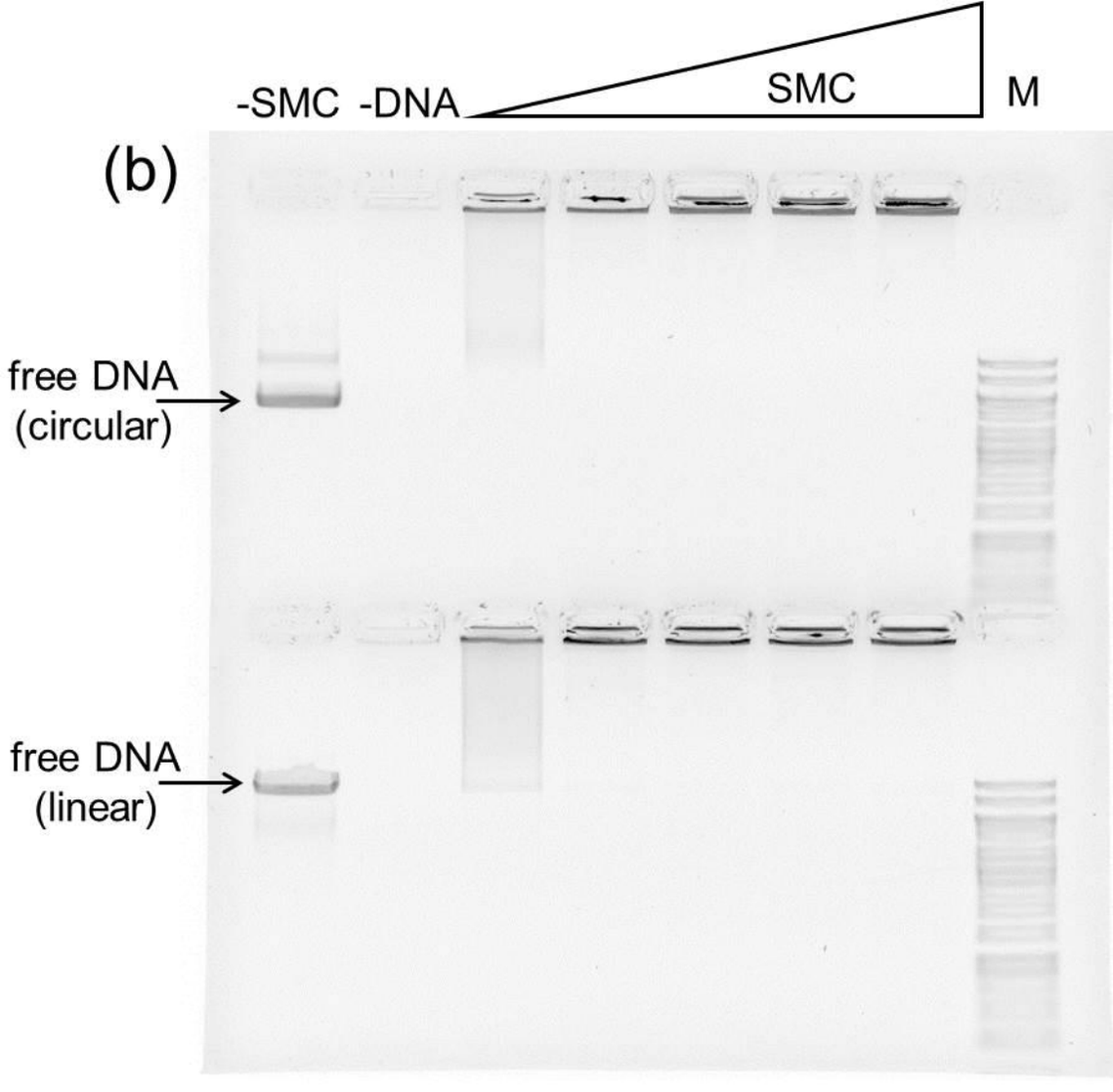
3,5


(a)
(b)
(c)
-SMC -ATP
+SMC -ATP
+SMC +ATP


(a)
M
ATP
-DNA -SMC
free DNA (circular)
free DNA (linear)
(b)
M
pH
-DNA -SMC
free DNA (circular)
free DNA (linear)
(c)
M
KCl
-DNA -SMC
free DNA (circular)
free DNA (linear)
(d)
M
MgCl2
-DNA -SMC
free DNA (circular)
free DNA (linear)


-SMC
SMC
-SMC
SMC
(a)
free DNA
(MTA)
free DNA
(GRIN2B)
ssDNA + dsDNA
dsDNA


-SMC
-DNA
SMC
M
(b)
free DNA
(circular)
free DNA
(linear)