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EXPERIMENTAL ARTICLES

Various Mechanisms of Flagella Helicity Formation in Haloarchaea

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Abstract—The genome of a halophilic archaeon *Haloarcula marismortui* carries two flagellin genes, *flaA2* and *flaB*. Previously, we demonstrated that the helical flagellar filaments of *H. marismortui* were composed primarily of flagellin FlaB molecules, while the other flagellin (FlaA2) was present in minor amounts. Mutant *H. marismortui* strains with either flagellin gene inactivated were obtained. It was shown that inactivation of the *flaA2* gene did not lead to changes in cell motility and helicity of the filaments, while the cells with inactivated *flaB* lost their motility and flagella synthesis was stopped. Two FlaB flagellin forms having different sensitivities to proteolysis were found in the flagellar filament structure. It is speculated that these flagellin forms may ensure the helical filament formation. Moreover, the flagella of a psychrotrophic haloarchaeon *Halorubrum lacusprofundi* were isolated and characterized for the first time. *H. lacusprofundi* filaments were helical and exhibited morphological polymorphism, although the genome contained a single flagellin gene. These results suggest that the mechanisms of flagellar helicity may differ in different halophilic archaea, and sometimes the presence of two flagellin genes, in contrast to *Halobacterium salinarum*, is not necessary for the formation of a functional helical flagellum.

Keywords: archaeal flagella, *Haloarcula marismortui, Halorubrum lacusprofundi*, filament, flagellin, biological motility

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Similar to bacteria, archaea move in liquid media by means of rotation of the helical flagella, which act as screw propellers. Despite the apparent similarity, bacterial and archaeal flagella differ fundamentally by their structure and mechanisms of assembly.

It is well known that flagellin, the protein forming the flagellar filaments of enterobacteria, possesses the unique ability to adopt either one of the two conformations, R or L, forming two types of protofilaments which differ in length and generate the filament twist upon association. Transitions between the different helical forms induced by changes in environmental conditions are due to various combinations of the R- and L-forms of flagellin within a filament and cooperative switches between them [1, 2].

Although archaeal flagellar filaments are also helical, they differ fundamentally from the bacterial ones by the mechanism of assembly, while archaeal flagellins exhibit no homology to bacterial flagellins [3]. As a rule, archaeal flagellar filaments are built of several types of flagellins encoded by different genes.

Earlier, we demonstrated that the presence of two flagellins, FlgA1 and FlgA2, is a prerequisite for formation of a helical flagellum in a haloarchaeon *Halobacterium salinarum*. The mutant strains with a single flagellin (FlgA1 or FlgA2) had straight nonfunctional filaments. We assumed that formation of the helical structure in archaea required two different proteins, which formed various types of protofilaments, rather than two conformations of a single flagellin [4, 5].

Later on, our attention was drawn to another halophilic archaeon *Haloarcula marismortui* which is strikingly different from *H. salinarum* in the arrangement of flagellin genes. In the genome of *H. salinarum*, flagellin genes are located on the chromosome within two operons, *flgA* and *flgB*, which contain five flagellin genes *flgA1, flgA2, flgB1–B3* [6]. Products of the five genes are present in the flagella with FlgA1 and FlgA2 being represented in comparable amounts and forming the major part of the filament [4]. In the genome of *H. marismortui*, two flagellin genes were discovered: one of them (*flaB*) was located on the chromosome, while the other one (*flaA2*) was located on the plasmid pNG100 [7]. Although we expected both flagellins to be present in the flagella, their major component was found to be flagellin FlaA2. Later, another stable phenotype of *H. marismortui* was identified, with the opposite situation: the filaments were made primarily of flagellin FlaB. The respective shares of the minor FlaA2 and FlaB flagellins in the filaments of the two phenotypes did not exceed 1%. Both types of filaments exhibited evident helical structure [8]. The mechanism of switching between the two flagella phenotypes is not clear yet.

We suggested that the filaments of *H. marismortui* were probably more similar to the bacterial type of organization when the helical supramolecular structure is based on the presence of two stable conforma-

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tional states of a single flagellin type, rather than two types of flagellin molecules, as in *H. salinarum* [8].

By now, full genome sequences of 17 halophilic archaea have been published, 16 of them contain flagellin genes. In three genomes (*Halalkalicoccus jeotgali, Halorhabdus tiamatea*, and *Halorubrum lacusprofundi*) a single copy of flagellin gene was revealed, while the other 13 genomes contained two to six genes encoding for flagellins. Remarkably, none of the "single-flagellin" microorganisms had ever demonstrated motility or flagellum synthesis [9–11].

We decided to explore whether the principle of helical filament formation we have discovered previously in *H. salinarum* is universal. One of the major goals of the present work was to study the role of the minor flagellin of *H. marismortui* in the filament helicity. For this purpose, mutant strains with either one of the two flagellin genes being inactivated were produced. The flagella of the halophilic archaeon *H. lacusprofundi*, which was previously considered nonmotile [11] and contains a single flagellin gene in the genome [12], were isolated and characterized.

MATERIALS AND METHODS

The strains used in the work included *Haloarcula marismortui* B-1809 (ATCC 43049, DSM 3752) and *Halorubrum lacusprofundi* B-1753 (ATCC 49239, DSM 5036) obtained from the All-Russian Collection of Microorganisms (VKM), Pushchino. *H. marismortui* cells were grown under moderate aeration at 42°C in a liquid medium containing the following (%): NaCl, 21.4; $MgSO_4 \cdot 7H_2O$, 1.7; KCl, 0.17; sodium citrate, 0.3; $CaCl_2$, 0.017; peptone, 0.85; yeast extract, 0.43; glucose, 0.34; and Tris-HCl, 0.52; pH 7.2. Filter-sterilized aqueous solution of microelements (1.7 mL) containing 0.0218% $MnCl_2 \cdot 7H_2O$ and 0.486% $FeCl_3 \cdot 7H_2O$ was added to 1 L of the medium after autoclaving. *H. lacusprofundi* was cultured at 32°C in a liquid medium containing the following (%): NaCl, 20; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 2; sodium citrate, 0.3; sodium glutamate, 0.1; casein hydrolysate, 0.5; and yeast extract, 0.5; pH 7.2. After autoclaving, the described microelement solution was added to the medium to the same final concentration.

Cell biomass of *H. marismortui* and *H. lacusprofundi*, as well as the preparations of their flagella, were obtained according to the techniques previously described [4, 8]. Protein SDS–PAGE was performed in 6–15% gels. Protein bands were stained with Coomassie G-250. Specific staining of glycoproteins with Schiff's reagent was performed directly in the gels [13]. Immunoblotting was performed with a ProtoBlot Western Blot Ap kit (Promega) according to the manufacturer's recommendations. Antisera were obtained by rabbit immunization with the purified filaments of the *H. marismortui* FlaB phenotype using the method described previously [14]. Limited trypsinolysis of *H. marismortui* filaments was carried out at room temperature in 10 mM Tris-HCl, pH 7.5, containing 20% NaCl and 10 mM $MgCl_2$. Flagellins in the buffer were mixed with the enzyme at various ratios. Trypsin and soybean trypsin inhibitor (Sigma) were preliminarily dissolved in the same buffer to the concentration of 1 mg/mL. Trypsinolysis was performed at the concentration of filaments ranging from 0.1 to 1 mg/mL and protein/enzyme ratio values of 5 to 500. Trypsinolysis was terminated by addition of the equimolar amount of soybean inhibitor.

To isolate the flagellin monomers, the precipitated flagella were dissolved in 10 mM Tris-HCl, pH 9.0, containing 8 M urea and 1% (vol/vol) Triton X-100. Flagellins were separated using an FPLC system equipped with a monoQ anion-exchange column in 0–1 M NaCl gradient in the buffer indicated above. To obtain the trypsinolysis-resistant flagellin form, FlaB-r, filaments of *H. marismortui* were heated at 65°C for 20 min at a concentration of 0.1 mg/mL. After they were cooled down to room temperature, trypsin was added in the amount of 1 mg per 5 mg filaments. After 10 h, trypsinolysis was terminated by addition of the equimolar amount of soybean inhibitor. The reaction mixture was then centrifuged for 30 min at 70000 rpm using a TLA-100 centrifuge (Beckman). Precipitated polymers contained both intact flagellin FlaB-r and the tryptic fragments. To remove these fragments, the precipitate was diluted with 10 mM Tris-HCl, pH 7.5, containing 7% NaCl and 10 mM $MgCl_2$, and centrifuged under the same conditions. Along with FlaB-r, the pellet contained low-molecular tryptic fragments containing hydrophobic N-terminal flagellin parts, while the fragments with C-terminal and central flagellin parts remained in the supernatant. The pellet was resuspended in 10 mM Tris-HCl, pH 9.0, containing 8 M urea and 1% (vol/vol) Triton X-100. Elution of FlaB-r flagellin and the tryptic fragments on a monoQ anion exchanger was performed at different NaCl concentration ranges, that is, 0.23–0.27 M and <0.17 M, respectively.

To inactivate the *flaA2* and *flaB* genes in *H. marismortui*, pWL102 plasmid used previously to inactivate the *H. marismortui* ribosomal operons [15] and kindly provided by Prof. P. Moore (Yale University, United States) was used. The plasmid is capable of replication in *E. coli* and contains the genes of resistance to ampicillin and mevinolin (an antibiotic acting against archaea). Among the additional advantages of the pWL102 vector is the absence of regions homologous to the *H. marismortui* genome. Therefore, inactivation plasmids on the basis of pWL102 may only incorporate into the genome at the region of flagellin genes. On the basis of pWL102 plasmid, constructions for insertional inactivation of genes *flaA2* and *flaB* were obtained. Regions of flagellin genes *flaA2* and *flaB* (825 and 734 bp long, respectively) were obtained using total DNA of *H. marismortui* as a template and the following primers:

5'-AGAAAAAAGCTTACGAGAACGAACGC-3' sense and 5'-TCCCACTTTCTAGATTTGTAATGGTAACCTC-3' antisense for inactivation of *flaA2* and

5'-TACAAATCTAGAAGACCGAGGCCAAGTG-3' sense and 5'-TCGTACTTGAAGCTTCAGTGATGTCAGAAC-3' antisense for inactivation of the *flaB* gene.

After purification and concentrating, PCR products were cloned into the pTZ57R/T vector from the InsTAclone PCR Cloning Kit (Fermentas, Lithuania). Intermediate cloning of PCR products in the pTZ57R/T vector and the following excision were performed to obtain sticky ends. Re-cloning of the fragments from vector pTZ57R/T to pWL102 was performed using the BamHI and EcoRI sites. Ligation mixture was used to transfect *E. coli* cells, which were then plated onto solid agar medium with ampicillin. During incubation, the colonies containing the required plasmid were formed. *H. marismortui* cells were transfected using polyethylene glycol according to the technique reported in [16] with some modifications. Due to rapid lysis of the cells in the process of spheroplast formation, the time after EDTA addition should be strongly controlled. Therefore, spheroplast formation was performed for each 2-mL aliquot independently. Spheroplasts were formed after addition of 20 μL 0.5 M EDTA during 10 min incubation at room temperature. Upon addition of DNA (approximately 2 μg), the mixture was incubated for 5 min, then an equal volume of 60% PEG-600 in unbuffered spheroplasting solution (wt/vol) was added. The cells were then incubated for 20 min at room temperature. To remove PEG, 1 mL culture medium was added to each tube and the culture was centrifuged during 5 min at 6000 rpm. The cell pellet was supplemented with 400 μL medium and incubated at 42°C for 4 h under moderate aeration. Then the cells were plated onto upper agar (0.5%) in petri dishes, which contained 2 μg/mL mevinolin. When incubated at 37°C, colonies were formed within 4 weeks. The constructions were used to transfect the cells resulting in formation of colonies growing on the selective medium. The in-*flaA2* and in-*flaB* mutants were grown in liquid medium at 37°C to OD_{600} of 1.0. Then the culture was stab-inoculated (aliquots of 2 μL) onto a plate with semisolid agar (0.25%) based on the medium for halophiles. Wild-type cells were used as the control. Growth was assessed after 5 days. Lysates of the mutant cells were analyzed by immunoblotting using polyclonal antibodies to the FlaB filaments of the wild type.

Mass spectrometry analysis was performed according to the method reported previously [8] on a liquid chromatography apparatus coupled with an ion trap LCQ DecaXP mass spectrometer (Thermo Finnigan, United States) equipped with a nanoelectrospray ion source. Elemental analysis of *H. marismortui* flagellins was carried out by I.P. Stolyarov (Kurnakov Institute of General and Inorganic Chemistry, Russian Academy of Sciences) on an upgraded CHN analyzer (Carlo Erba Instruments, Italy). Bovine serum albumin was used as the control.

For electron microscopy studies, a JEM-100c (JEOL, Japan) microscope was used. Samples were applied to formvar-coated electron microscopy copper grids and stained with 2% uranyl acetate.

RESULTS

1. Obtaining the Mutant H. marismortui Strains with Inactivated Flagellin Genes

As was observed earlier [8], relative content of the FlaA2 flagellin in the filaments of the FlaB phenotype of *H. marismortui* did not exceed 1%. Inactivation of the *flaB* gene was found to result in complete loss of motility and termination of flagella synthesis (Figs. 1a and 1b). Therefore, the minor flagellin FlaA2 could not compensate for the absent FlaB. Inactivation of *flaA2* gene did not lead to changes in cell motility, if compared to the wild type or morphology of the filaments, which retained the helical shape (Figs. 1c and 1d). These results evidence that a single flagellin gene is sufficient for the formation of functional helical filaments in *H. marismortui*.

2. Identification of Two Flagellin FlaB Forms in H. marismortui.

According to differential scanning microcalorimetry under natural salinity conditions (20% NaCl, 10 mM $MgCl_2$, pH 7.5), FlaB filament melting curve contains two peaks of heat absorption at 55°C (peak 1) and 84°C (peak 2) [8]. Melting of the flagellin domains is irreversible and is accompanied by loss of resistance to proteolysis. Treatment with trypsin after heating to 90°C resulted in rapid flagellin cleavage into a number of fragments,with no intact protein remaining (Fig. 2). A somewhat different picture of trypsinolysis was observed for partially denatured (heated to 65°C) filaments: most of the initial flagellin (approximately 80%) was cleaved into fragments within minutes, while the rest (20%) remained intact even after 24-h trypsinolysis (Fig. 2). Therefore, two forms of flagellin FlaB comprised the filaments, namely, FlaB-r (resistant to proteolysis) and FlaB-s (sensitive to proteolysis), which differed in the quaternary structure and were characterized by different availability to trypsin attack.

We assumed that FlaB-s and FlaB-r may be analogous to the R- and L-forms of the bacterial flagellin, which ensure formation of a helical filament by the molecules of a single protein. In this connection, it was of interest to find out whether the difference between FlaB-r and FlaB-s was retained upon the filament dissociation. In other words, did FlaB-r and FlaB-s differ by post-translational modifications (e.g., two glycoforms) or were they completely identical, with the difference between them emerging only within a filament? Comparison of electrophoretic

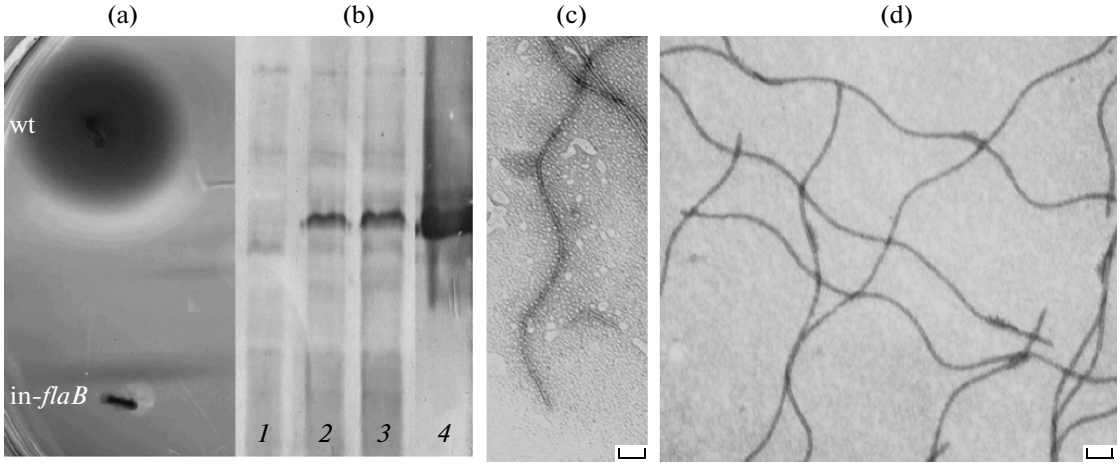

Fig. 1. Mutant *H. marismortui* strains with inactivated flagellin genes. (a) Growth of *H. marismortui* FlaB-phenotype point inocula on a semi-liquid medium containing 0.25% agar: wild type (top) and mutant strain with inactivated *flaB* gene (bottom). (b) Immunoblotting of *H. marismortui* of FlaB-phenotype preparations: *1*, lysate of cells with inactivated *flaB* gene, *2*, lysate of cells with inactivated *flaA2* gene, *3*, wild type, *4*, FlaB filaments. Polyclonal rabbit antibodies against purified FlaB-filaments were used. Electron microscopy images of (c) FlaB filaments of wild type and (d) filaments isolated from cells with inactivated *flaA2* gene. Scale bar corresponds to 0.1 μm.

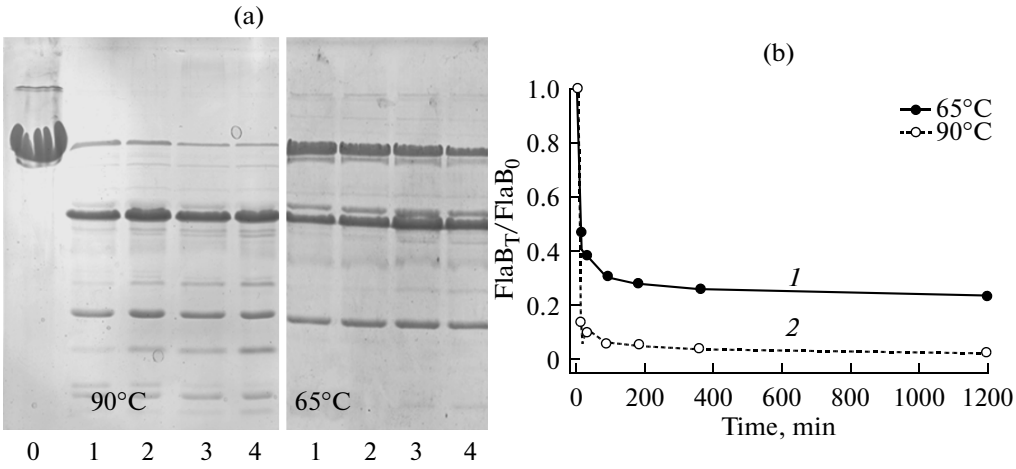

Fig. 2. Time course of trypsinolysis of *H. marismortui* FlaB filametns after heating to 90°C and 65°C in 50 mM Tris-HCl, pH 7.5, containing 20% NaCl and 10 mM $MgCl_2$. Trypsinolysis was performed at 25°C, protein-to-enzyme ratio was 5 : 1. (a) Electrophoresis picture of filament preparations before trypsinolysis (lane 0), and after 15, 30, 180, and 1200 min of trypsinolysis (lanes 1–4 respectively). (b) Changes in relative content of intact flagellin in filament preparations in the course of trypsinolysis: *1*, after 65°C, and *2*, after 90°C.

mobility of FlaB-r and FlaB-s did not reveal any noticeable differences. At the same time, results of anion exchange chromatography demonstrated that, under denaturing conditions, FlaB-r and FlaB-s were not identical. Figure 3 shows elution profiles of dissociated FlaB filaments (FlaB-r + FlaB-s) and FlaB-r on a monoQ anion exchanger in 10 mM Tris-HCl buffer, pH 9.0 containing 1% Triton X-100 and 8 M urea. It should be noted that for complete dissociation of archaeal filaments, strong detergents such as Triton X-100 were required to prevent formation of hydrophobic contacts in the proteins. Elution profiles had intricate shape: apparently, both FlaB-r and FlaB-s were not homogeneous and each of them could be separated into several subforms.

3. Isolation and Some Properties of H. lacusprofundi Filaments

The strain *Halorubrum lacusprofundi* B-1753 (initial name *Halobacterium lacusprofundi* ACAM 34) was isolated from a relict hypersaline Deep Lake in Antarctica [11, 17]. Due to high salinity, the lake never freezes and the surface temperature changes from

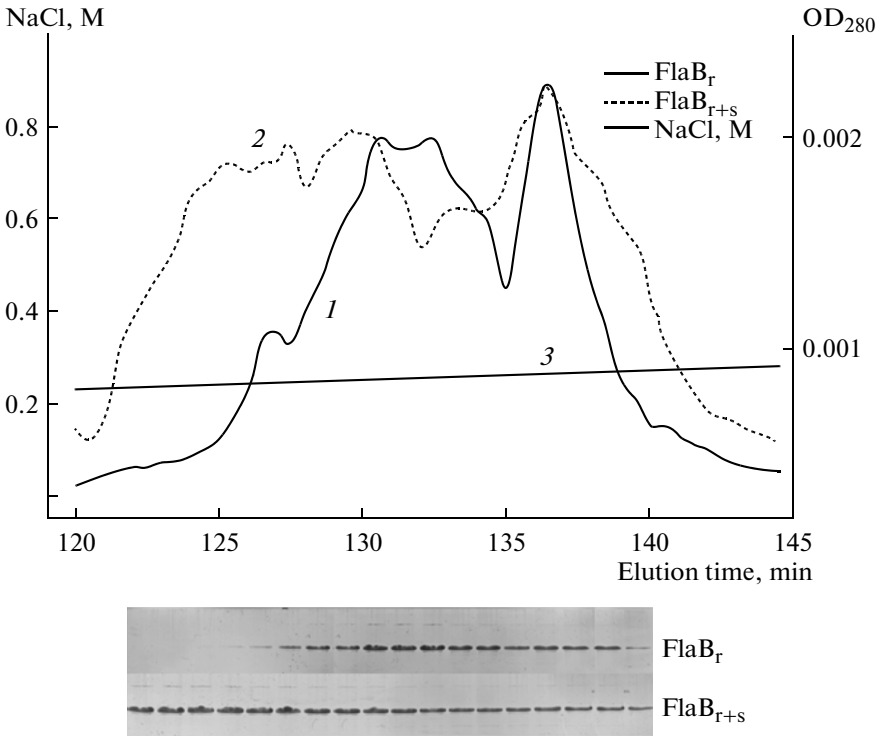

Fig. 3. Elution profiles obtained upon fractionation of flagellin preparations of *H. marismortui* FlaB phenotype on an anion exchanger in 10 mM Tris-HCl buffer, pH 9.0, containing 8M urea and 1% Triton X-100: *1*, FlaB-r, *2*, FlaB-r + FlaB-s, *3*, NaCl molarity. Flow rate 0.3 mL/min. Electrophoresis picture of the flagellin-containing fractions 122–140 (fraction volume 0.3 mL) is presented at the bottom of the panel.

–20°C to +10°C during the year. Under laboratory conditions, *H. lacusprofundi* cells may grow at temperatures ranging from –1°C to +44°C (temperature optimum at +33°C) [11]. According to the initial taxonomic description [11] and current opinion (S. DasSarma, personal communication, 2010) the strain is nonmotile and has no flagella (despite the presence of the relevant genes in its genome). After several cycles of selection of *H. lacusprofundi* cells in semisolid (0.19%) agar, we managed to obtain the cells with noticeable motility and to isolate the filaments using the method used previously to obtain flagella of other haloarchaea [4, 6, 8, 18]. According to electron microscopy data (Fig. 4b), most of the filaments were helical. Several filament forms were observed differing by the helix pitch distance and diameter, as well as circles resembling the well-known polymorphous forms of bacterial filaments (the so-called normal, curly, coiled, etc.) [19]. Filaments were homogenous in thickness (10–11 nm). The structures resembling the hooks or basal bodies of bacterial flagella were not observed.

SDS-PAGE of *H. lacusprofundi* filaments (Fig. 4a) produced a single major band, corresponding to the molecular mass of about 50 kDa, and an additional band of a dimer (which is typical of archaeal flagellins [18]). Mass spectrometry analysis (data not shown) confirmed that the isolated protein was the *H. lacusprofundi* flagellin. It should be noted that molecular masses of flagellins and other proteins of extremely halophilic archaea determined electrophoretically are often much higher than the true values (23.6 kDa for the flagellin of *H. lacusprofundi*) due to increased content of carbonic acids and posttranslational modifications leading to abnormal behavior in SDS-PAGE [6, 8, 18].

Anion exchange chromatography applied with electrophoresis (as in the case of *H. marismortui*) made it possible to reveal several forms of flagellin characterized by close electrophoretic mobility values but differing by chromatographic characteristics. Figure 5 demonstrates the elution profile of *H. lacusprofundi* filaments on a monoQ anion exchange column in 10 mM Tris-HCl buffer, pH 9.0, containing 1% Triton X-100 and 8 M urea. We found that the filaments contained at least two major forms of flagellin. Relative content of the flagellin forms in the filaments was evaluated by the ratio of the relevant peak areas on the chromatograms. The major flagellin form (peak on the right) comprised about 40% of the total protein.

4. Glycosylation of Flagellins of H. lacusprofundi and H. marismortui

Flagellins of many archaea are known to be glycoproteins [3]. It was shown for methanogenic archaea

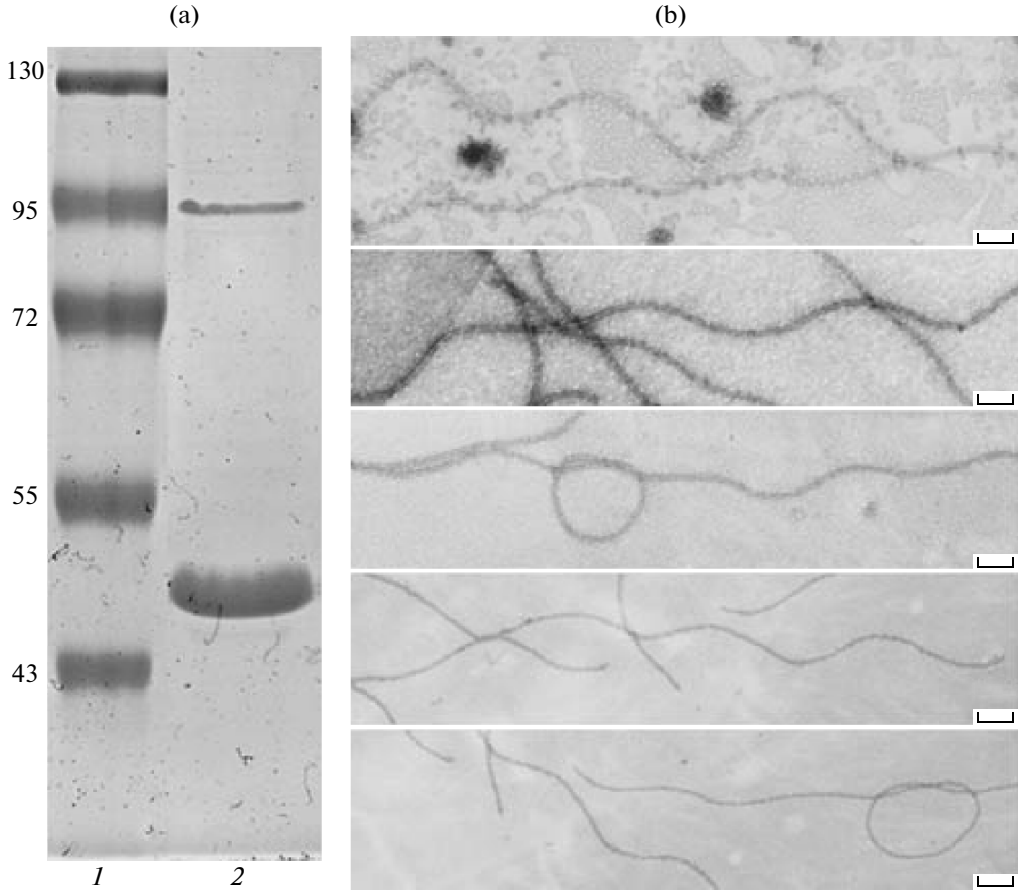

Fig. 4. Isolation of *H. lacusprofundi* filaments. (a) SDS-PAGE: *1*, protein weight markers, kDa; *2*, filament preparation obtained upon precipitation with polyethylene glycol and purification in cesium chloride gradient. (b) Electron microscopy images of the filaments. Scale bar corresponds to 0.1 μm.

that impairment of glycosylation could lead to arrest of flagellum assembly and loss of motility [20]. It is assumed that glycosylation is used for additional stabilization of flagellin structure under the extreme conditions typical for archaeal habitats. Earlier, the structures of oligosaccharides comprising the flagellins of *H. salinarum, Methanococcus voltae*, and *Methanococcus maripaludis* had been identified. Until now, the only type of glycosylation observed in archaeal flagellins was N-glycosylation with sugar moieties bound to the NH group of asparagine [3] and Asn-X-Ser(Thr) sequence, with X being any amino acid except for proline marking the glycosylation sites.

The flagellin of *H. lacusprofundi* contained six characteristic N-glycosylation sites while amino acid sequences of the flagellins FlaB and FlaA2 of *H. marismortui* contained no such sites. Glycosylation of the flagellin of *H. lacusprofundi* was confirmed using specific staining with Schiff's reagent (Fig. 6). The observed flagellin forms of *H. lacusprofundi* probably corresponded to various glycoforms of the flagellin, and at the same time represented the analogues of the L- and R-conformations of bacterial flagellin necessary for assembly of a helical filament. One may not exclude that different types of glycosylation (or other post-translational modifications) may fix these states.

The bands corresponding to the flagellins of *H. marismortui* in polyacrylamide gel were also stained with Schiff's reagent (Fig. 6a), although the color intensity decreased significantly upon thorough washing with water, in contrast to the flagellin of *H. lacusprofundi* and the flagellins of *H. salinarum* used as positive controls (Fig. 6b). Additional confirmation of glycosylation of *H. marismortui* flagellins may be the data of CHN-analysis: experimental C/N values for FlaB and FlaA2 flagellins were the same (3.50 ± 0.08) which was significantly higher than the values calculated for the relevant amino acid sequences (3.200 for FlaB and 3.204 for FlaA2). Since amino acid residues of the flagellins contained no canonical sites of N-glycosylation, we assume that either N-glycosylation at an uncommon site [21] or O-glycosylation occurred. The flagellin FlaB forms mentioned above may correspond to different glycoforms.

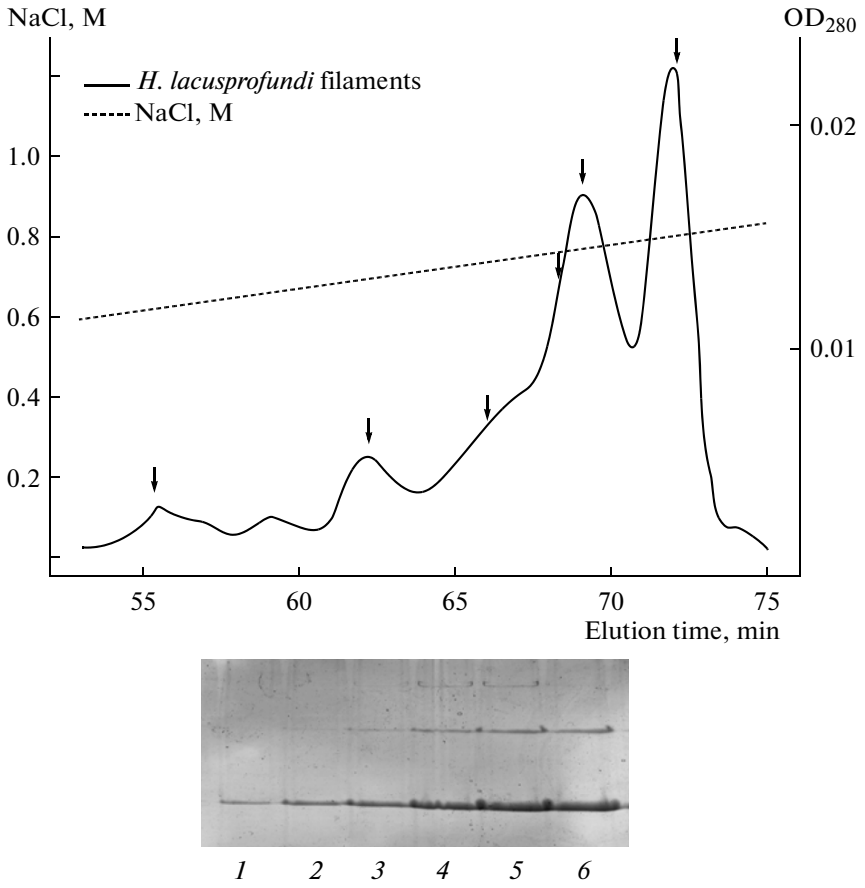

Fig. 5. Elution profile obtained upon fractionation of *H. lacusprofundi* flagellin forms on an anion exchanger in 10 mM Tris-HCl, pH 9.0, containing 8 M urea and 1% Triton X-100; flow rate 0.3 mL/min. Electrophoresis picture of flagellin-containing fractions, indicated with arrows, is presented at the bottom of the panel (fraction volume 0.3 mL).

DISCUSSION

The presence of several copies of flagellin genes is more typical of archaeal genomes than of bacterial ones. Today over 100 archaeal full-genome sequences have been identified with over 65% of the deciphered genomes containing the genes encoding flagellins. As a rule, archaeal genomes contain several copies of flagellin genes organized into 1–3 clusters. Each cluster may contain one to eight flagellin genes (http://archaea.ucsc.edu/genomes/archaea/).

In the genomes of the *Crenarchaeota* the number of flagellin genes does not exceed two, while the presence of a single flagellin gene is probably typical of this archaeal group.

Most known genomes of the *Euryarchaeota* contain multiple (two to nine) copies of flagellin genes. Among 58 of euryarchaeotic genomes containing flagellin genes, only 7 (*Ferroglobus placidus, Methanosarcina barkeri* str. Fusaro, *Thermococcus barophilus*, and *Thermococcus sibiricus*, as well as the three haloarchaeal genomes mentioned above) contain a single copy of the flagellin gene. Remarkably, motility and the presence of flagella were observed only in two of the single-flagellin euryarchaeota, namely, *F. placidus* and *T. barophilus*. In the *Euryarchaeota*, flagella comprising the products of several flagellin genes probably possess evolutionary benefits.

Some of the known archaeal flagellins are not the major structural proteins of the filament. In a number of methanogenic archaea, the structures morphologically similar to the hooks of bacterial flagella were observed [3]. By the example of *Methanococcus voltae* and *Methanococcus maripaludis* it was demonstrated that a segment of archaeal hook was built of the molecules of flagellin FlaB3, while the other two flagellins (FlaB1 and FlaB2) were the major components of the filament [22, 23]. Inactivation of the *flaB3* gene did not lead to impaired filament synthesis and considerable changes in cell motility in semisolid agar. Phase contrast microscopy, however, revealed abnormal motility in the mutant cells (circular movement). Inactivation of each of the genes *flaB1* and *flaB2* led to the loss of cell motility and termination of the synthesis of flagella (including the hook) [23]. For other archaea, no structures similar to the hooks of *Methanococcus* were observed in native flagella. There is some evidence that differentiation of one of the flagel-

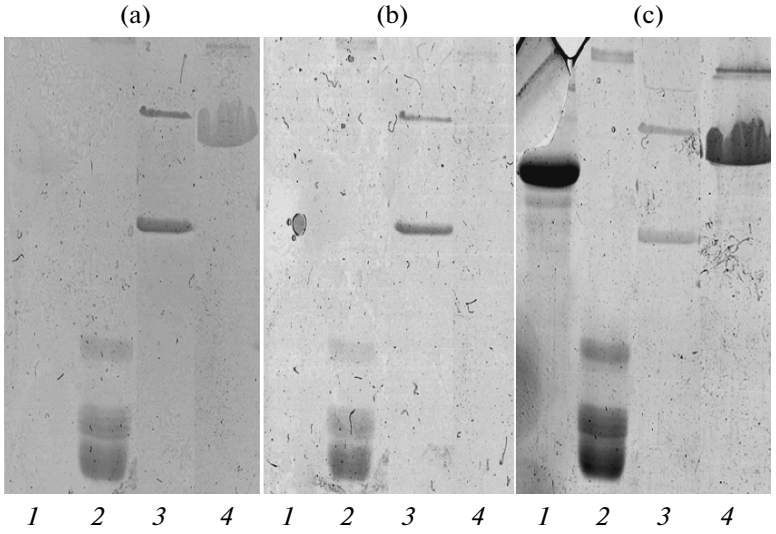

Fig. 6. Flagellin staining with Schiff's reagent. After staining, 14% polyacrylamide gel was washed with water for (a) 1 h and (b) overnight; (c) the same gel stained with Coomassie G-250. *1*, bovine serum albumin (negative control), *2*, *H. salinarum* flagellins (positive control), *3*, *H. lacusprofundi* filaments, and *4, H. marismortui* FlaB filaments.

lins into the hook protein with an individual structural function is a relatively late evolutionary acquisition of the genus *Methanococcus* and is not typical of other archaea.

Earlier, we have studied the structure of the filaments of a haloarchaeon *H. salinarum*. It was demonstrated that the mutant cells containing only the *flgA1* or *flgA2* gene possessed straight flagella. We assumed that to form the helical structure of the filament, archaea employ two different proteins, forming different types of protofilaments, rather than two conformations of the same protein [4]. Another subject of our studies, a halophilic archaeon *H. marismortui*, has two flagellin genes located in different replicons. Although we expected to see both flagellins in the filament structure, the major component was a single flagellin. The share of the minor one did not exceed 1% [8]. Our results demonstrate that, in contrast to *H. salinarum*, the functional helical filaments may be built of a single flagellin.

This is the first experimental demonstration of the presence of two forms of flagellin FlaB in the filaments. We assumed that FlaB-r and FlaB-s probably corresponded to two conformational states of a single flagellin, which ensure the formation of a helical filament. During the filament assembly, flagellin conformational state is probably given by the co-assembly folding upon the interaction with protein partners and is further fixed by glycosylation.

We were the first to isolate and characterize the flagella of a halophilic archaeon *H. lacusprofundi*, which contains a single flagellin gene in its genome and was previously considered nonmotile. It was demonstrated that the absence of additional flagellin genes did not hamper formation of the helical filaments. Several flagellin forms were identified in the filament, probably representing different glycoforms of the protein. Earlier, by the example of a crenarchaeon *Sulfolobus solfataricus*, it was demonstrated that the absence of additional flagellin genes did not hamper formation of the functional helical flagella [24]. Analysis of protein content of the *Sulfolobus* flagellar filaments by electrophoresis demonstrated the presence of two bands which apparently corresponded to two glycoforms of the flagellin [25]. Interestingly, in contrast to other haloarchaea, we observed several types of *H. lacusprofundi* filaments. Earlier, it was demonstrated that impairment of filament glycosylation in *Pseudomonas syringae* had a similar effect [26]. One may not exclude that the observed polymorphism of filaments in *H. lacusprofundi* was probably triggered by suboptimal proportions between the flagellin forms under laboratory cultivation conditions.

Gradually, it becomes apparent that structural organization of archaeal filaments is much more diverse than it has been considered previously. Our results indicate that the filaments of *H. marismortui* and *H. lacusprofundi*, in some sense, are closer to the bacterial type of structural organization, when the assembly of a helical supramolecular structure is ensured by the presence of two stable conformational states of a single flagellin type. At the same time, the presence of posttranslational modifications (glycosylation) may be required for helicity of the single-flagellin archaeal filaments.

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REFERENCES

1. Calladine, C.R., Construction of Bacterial Flagellar Filaments, and Aspects of Their Conversion to Different Helical Forms, *Symp. Soc. Exp. Biol.*, 1982, vol. 35, pp. 33–51.
2. Maki-Yonekura, S., Yonekura, K., and Namba, K., Conformational Change of Flagellin for Polymorphic Supercoiling of the Flagellar Filament, *Nat. Struct. Mol. Biol.*, 2010, vol. 17, pp. 417–422.
3. Ng, S.Y.M., Chaban, B., and Jarrell, K.F., Archaeal Flagella, Bacterial Flagella and Type IV Pili: A Comparison of Genes and Posttranslational Modifications, *J. Mol. Microbiol. Biotechnol.*, 2006, vol. 11, pp. 167–191.
4. Tarasov, V.Y., Pyatibratov, M.G., Tang, S.-L., Dyall-Smith, M., and Fedorov, O.V., Role of Flagellins from A and B Loci in Flagella Formation of *Halobacterium salinarum, Mol. Microbiol.*, 2000, vol. 35, pp. 69–78.
5. Tarasov, V.Y., Pyatibratov, M.G., Beznosov, S.N., and Fedorov, O.V., On the Supramolecular Organization of the Flagellar Filament in Archaea, *Doklady Biochem. Biophys.*, 2004, vol. 396, pp. 203–205.
6. Gerl, L. and Sumper, M., Halobacterial Flagellins Are Encoded by a Multigene Family. Identification of Five Flagellin Genes, *J. Biol. Chem.*, 1988, vol. 263, pp. 13246–13251.
7. Baliga, N.S., Bonneau, R., Facciotti, M.T., Pan, M., Glusman, G., Deutsch, E.W., Shannon, P., Chiu, Y., Weng, R.S., Gan, R.R., Hung, P., Date, S.V., Marcotte, E., Hood, L., and Ng, W.V., Genome Sequence of *Haloarcula marismortui*: A Halophilic Archaeon from the Dead Sea, *Genome Res.*, 2004, vol. 14, pp. 2221–2234.
8. Pyatibratov, M.G., Beznosov, S.N., Rachel, R., Tiktopulo, E.I., Surin, A.K., Syutkin, A.S., and Fedorov, O.V., Alternative Flagellar Filament Types in the Haloarchaeon *Haloarcula marismortui, Can. J. Microbiol.*, 2008, vol. 54, pp. 835–844.
9. Roh, S.W., Nam, Y.D., Chang, H.W., Sung, Y., Kim, K.H., Oh, H.M., and Bae, J.W., *Halalkalicoccus jeotgali* sp. nov., a Halophilic Archaeon from Shrimp Jeotgal, a Traditional Korean Fermented Seafood, *Int. J. Syst. Evol. Microbiol.*, 2007, vol. 57, pp. 2296–2298.
10. Antunes, A., Taborda, M., Huber, R., Moissl, C., Nobre, M.F., and Da Costa, M.S., *Halorhabdus tiamatea* sp. nov., a Non-Pigmented, Extremely Halophilic Archaeon from a Deep-Sea Hypersaline Anoxic Basin of the Red Sea, and Emended Description of the Genus *Halorhabdus, Int. J. Syst. Evol. Microbiol.*, 2008, vol. 58, pp. 215–220.
11. Franzmann, P.D., Stackebrandt, E., Sanderson, K., Volkman, J.K., Cameron, D.E., Stevenson, P.L., McMeekin, T.A., and Burton, H.R., *Halobacterium lacusprofundi*, sp. nov., a Halophilic Bacterium Isolated from Deep Lake, Antarctica, *Syst. Appl. Microbiol.*, 1988, vol. 11, pp. 20–27.
12. DasSarma, S. (Ed.), *Halorubrum lacusprofundi* Genome Database, 2002–2011. http://halo4.umbi.umd.edu/cgi-bin/haloweb/hla.pl
13. Doerner, K.C. and White, B.A., Detection of Glycoproteins Separated by Nondenaturing Polyacrylamide Gel Electrophoresis Using the Periodic Acid-Schiff Stain, *Anal. Biochem.*, 1990, vol. 187, pp. 147–150.
14. Southam, G., Kalmokoff, M.L., Jarrell, K.F., Koval, S.F., and Beveridge, T.J., Isolation, Characterization and Cellular Insertion of the Flagella from Two Strains of the Archaebacterium *Methanospirillum hungatei, J. Bacteriol.*, 1990, vol. 172, pp. 3221–3228.
15. Tu, D., Blaha, G., Moore, P.B., and Steitz, T.A., Gene Replacement in *Haloarcula marismortui*: Construction of a Strain with Two of Its Three Chromosomal rRNA Operons Deleted, *Extremophiles*, 2005, vol. 9, pp. 427–435.
16. Cline, S.W., Lam, W.L., Charlebois, R.L., Schalkwyk, L.C., and Doolittle, W.F., Transformation Methods for Halophilic Archaebacteria, *Can. J. Microbiol.*, 1989, vol. 35, pp. 148–152.
17. McGenity, T.J. and Grant, W.D., Transfer of *Halobacterium saccharovorum, Halobacterium sodomense, Halobacterium trapanicum* NRC 34021 and *Halobacterium lacusprofundi* to the Genus *Halorubrum* gen. nov., as *Halorubrum saccharovorum* comb. nov., *Halorubrum sodomense* comb. nov., *Halorubrum trapanicum* comb. nov., and *Halorubrum lacusprofundi* comb. nov., *Syst. Appl. Microbiol.*, 1995, vol. 18, pp. 237–243.
18. Fedorov, O.V., Pyatibratov, M.G., Kostyukova, A.S., Osina, N.K., and Tarasov, V.Y., Protofilament as a Structural Element of Flagella of Haloalkalophilic Archaebacteria, *Can. J. Microbiol.*, 1994, vol. 40, pp. 45–53.
19. Berg, H.C., The Rotary Motor of Bacterial Flagella, *Annu. Rev. Biochem.,* 2003, vol. 72, pp. 19–54.
20. Chaban, B., Voisin, S., Kelly, J., Logan, S.M., and Jarrell, K.F., Identification of Genes Involved in the Biosynthesis and Attachment of *Methanococcus voltae* N-Linked Glycans: Insight into N-Linked Glycosylation Pathways in Archaea, *Mol. Microbiol.*, 2006, vol. 61, pp. 259–268.
21. Zeitler, R., Hochmuth, E., Deutzmann, R., and Sumper, M., Exchange of Ser-4 for Val, Leu or Asn in the Sequon Asn-Ala-Ser Does Not Prevent N-Glycosylation of the Cell Surface Glycoprotein from *Halobacterium halobium, Glycobiology*, 1998, vol. 8, pp. 1157–1164.
22. Bardy, S.L., Mori, T., Komoriya, K., Aizawa, S.-I., and Jarrell, K.F., Identification and Localization of Flagellins FlaA and FlaB3 within the Flagella of *Methanococcus voltae, J. Bacteriol.*, 2002, vol. 184, pp. 5223–5233.
23. Chaban, B., Ng, S.Y.M., Kanbe, M., Saltzman, I., Nimmo, G., Aizawa, S.-I., and Jarrell, K.F., Systematic Deletion Analyses of the *fla* Genes in the Flagella Operon Identify Several Genes Essential for Proper Assembly and Function of Flagella in the Archaeon, *Methanococcus maripaludis, Mol. Microbiol.*, 2007, vol. 66, pp. 596–609.
24. Szabó, Z., Sani, M., Groeneveld, M., Zolghadr, B., Schelert, J., Albers, S.V., Blum, P., Boekema, E.J., and Driessen, A.J., Flagellar Motility and Structure in the Hyperthermoacidophilic Archaeon *Sulfolobus solfataricus, J. Bacteriol.*, 2007, vol. 189, pp. 4305–4309.
25. Faguy, D.M., Bayley, D.P., Kostyukova, A.S., Thomas, N.A., and Jarrell, K.F., Isolation and Characterization of Flagella and Flagellin Proteins from the Thermoacidophilic Archaea *Thermoplasma volcanium* and *Sulfolobus shibatae, J. Bacteriol.*, 1996, vol. 178, pp. 902–905.
26. Taguchi, F., Shibata, S., Suzuki, T., Ogawa, Y., Aizawa, S., Takeuchi, K., and Ichinose, Y., Effects of Glycosylation on Swimming Ability and Flagellar Polymorphic Transformation in *Pseudomonas syringae* pv. *tabaci,* 6605, *J. Bacteriol.,* 2008, vol. 190, pp. 764–768.