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EXPERIMENTAL ARTICLES

On the Multicomponent Nature of *Halobacterium salinarum* Flagella

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Abstract—Filaments of the flagellum of the halophilic archaeon *Halobacterium salinarum* consist of five flagellins: A1, A2, B1, B2, and B3, which are encoded by five genes localized in tandem in two *flgA* and *flgB* operons. While the role of flagellins A1 and A2 has been determined, the role of the proteins, *B* operon products, is still unclear. A mutant strain of *H. salinarum* with deleted *A* and *B* flagellin genes (Δ*flgA*Δ*flgB*) has been obtained for the first time. This strain has been used to create and analyze the strains carrying only individual *B1* or *B3* flagellin genes. Cells of the Δ*flgA*Δ*flgB* strain were shown to have short filamentous formations, 7–8 nm thick, which we have named as X-filaments. It has been shown that X-filaments consist of a protein immunologically related to flagellins A and B. Expression of the *B1* and *B3* genes is suppressed in the absence of *A1, A2*, and *B2*. It has been shown that flagellins B1 and B3 cannot be substituted for flagellin B2 upon the formation of a curved hook-like structure, which serves as a connecting element between the flagellar filament and the motor axis. The multicomponent nature of flagella is discussed in the light of their possible involvement in other cell processes besides providing motility.

Key words: archaeal flagellum, *Halobacterium salinarum*, filament, flagellin, motility of archaea.

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Motility of cells is typical of representatives of all three domains of living organisms: Eucarya, Bacteria, and Archaea. Carl Woese assigned archaea to a separate taxonomic domain on the basis of comparative analysis of 16S rRNA of different organisms; further studies confirmed the validity of this division [1]. Archaea include extremely halophilic, methanogenic, thermoacidophilic (*Thermoplasma*), sulfur-dependent thermophilic, and hyperthermophilic organisms. The archaea are similar to bacteria in a number of morphological, biochemical and physiological properties but concurrently have features typical of eukaryotes. At the same time, the archaea possess unique properties inherent only to representatives of this group [2]. In particular, studies of archaeal flagella disproved the original hypothesis that the motility apparatus of archaea was analogous to the bacterial one. For example, it was shown for bacteria that the outer spiral part of the flagellum (filament) is assembled from a single protein (flagellin), whereas in archaea this supramolecular structure is usually multicomponent. This was first revealed by the results of determination of the protein composition of flagellar filaments of *Halobacterium salinarum* (previously *Halobacterium halobium*) [3]. Then it was demonstrated that the amino acid sequences of *H. salinarum* flagellins has no homology with bacterial flagellins [4, 5]. Later, other differences were found, e.g., in the direction of the flagellum filament assembly and in the presence of signal sequences and posttranslational modifications which are missing in bacterial flagellins. Comparison of the known genome sequences of bacteria and archaea showed that genomes of the latter lack homologues of other most important proteins of the bacterial motor. At the same time, conservative genes were found in the immediate proximity of the genes of archaeal flagellins. They were designated as *fla* genes (*flaC*, *D*, *E*, *F*, *G*, *H*, *I*, and *J*) as their products were supposed to be responsible for the synthesis and functioning of the flagellar motor of archaea. While having no noticeable homology with bacterial flagellins, the archaeal flagellins show a certain similarity to the proteins of bacterial type-IV pili; products of the *flaI* and *flaJ* genes also demonstrate homology with the proteins of type-IV pili synthesis systems [6]. The collected data make it possible to consider the archaeal flagellum as a unique structure of biological motility different from bacterial flagella [6–8]. However, at present there are no reliable concepts of the process of flagellum assembly or the mechanism of its functioning.

At present, the complete genome sequences of many archaea are known. The number of flagellin genes is usually two to six. The genes may be localized either within a single locus or on chromosome regions distant

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from each other. *Sulfolobus* sp. genomes with a single flagellin gene are exceptional in this respect. *H. salinarum* has the highest number of flagellin genes (six) of the three motile halophilic archaea: *H. salinarum, Haloarcula marismortui*, and *Natronomonas pharaonis.* The genome of *N. pharaonis* contains three flagellin genes localized in tandem. *H. marismortui* was shown to have only two full-fledged flagellin genes localized on different replicons [6]. Thus, the number of genes that encode the proteins classified as flagellins may greatly differ even in related species. The works on the knock-out of flagellin genes in several archaea [7, 9–11] made it possible to define the potential functions of some flagellins and to show that in these cases the multicomponent composition of a flagellum was required to maintain its spiral form. However, there is still the question of why six flagellin genes are present in the genome of one archaeal species, whereas two are sufficient for another closely related archaeon.

The flagellum of the halophilic archaeon *H. salinarum* consists of five flagellins: A1, A2, B1, B2, and B3, which are encoded by five different genes localized in tandem in two operons: *flgA* (*flgA1*, *flgA2* genes) and *flgB* (*flgB1*, *flgB2*, *flgB3* genes). The sixth flagellin gene *flgXXX*, localized apart from the *A* and *B* loci, was identified from the complete genome sequence of *H. salinarum* [12]. The product of this gene has not been found as yet as a flagellum component or among cellular proteins. The amino acid sequence of the *flgXXX* gene product is homologous to other flagellins and approximately equal to them in size. At the same time, flagellins A and B are less different from each other than each of them differs from protein XXX (e.g., the signal sequence identical for the five A and B flagellins is critically different from that of flagellin XXX).

The strains previously obtained by our team carried only one of the two *flgA* genes; they had straight flagella and their motility was much less as compared with the wild-type cells. The cells of strain Δ*flgB* (*B* operon deleted) had extensive spiral filaments analogous to the filaments of wild-type flagella. At the same time, strain Δ*flgA* (*A* operon deleted) had short curved filaments all over the cell surface. We hypothesized that B flagellins formed a flagellar hook region [9, 11].

In this work, we have obtained and analyzed a mutant strain Δ*flgA*Δ*flgB* of *H. salinarum* (all *A*- and *B* flagellin genes deleted) for the first time. By means of specially constructed plasmids, new strains carrying individual *B1*- or *B3* flagellin genes have been obtained from this strain and analyzed.

The objective of our studies was to ascertain the role of the products of flagellin genes of *H. salinarum*, which has the highest number of respective genes among the archaea with known genomes.

MATERIALS AND METHODS

Strain *Halobacterium salinarum* R1M1 (kindly provided by S.I. Bibikov from the Belozersky Institute of Physicochemical Biology, Moscow State University) and the mutant strain of *H. salinarum* with deleted *flgA* operon (Δ*flgA*) constructed by V.Yu. Tarasov [9] were used in the work. Strain *E. coli* DH5α *supE44* Δ*lacU*169 (φ80 *lacZ* ΔM15) *hsdR*17 *recA1 endA1 gyrA*96 *thi-1 relA1* was used for genetic engineering manipulations. *H. salinarum* cells were grown in a liquid medium of the following composition: 25% NaCl; 0.2% KCl; 2% $Mg_2SO_4 \cdot 7H_2O$; 0.3% Na citrate; 0.5% tryptone; 0.2% yeast extract; pH 7.5.

Chromosomal DNA of *H. salinarum* was isolated as described in [13]. Polymerase chain reaction and agarose gel electrophoresis of the obtained fragments of nucleic acids were as in [9]. Primers were synthesized by Sintol Company (Moscow). The *B*-flagellin operon with flanking regions was obtained using primers: (5'-GTCCGCCGGAAGCTTCGGATCGAGATC-3') and (5'-CTCCGACTCTAGATTCTCCACGAACGC-3'). Annealing was carried out for 1 min; the temperature of annealing was selected experimentally by the maximal yield of specific reaction products and varied within 55 to 65°C.

The plasmids used in the work were based on plasmid pNX constructed by V.Yu. Tarasov on the basis of plasmid pUC19 [9] with the introduced genes *NovR* (the gene of resistance to novobiocin) from plasmid pMDS20 [14] and *bgaH* (the gene of halophilic β-galactosidase) from plasmid pMLH32 [15]. Plasmid pNXΔB was used for deletion of *B*-flagellin operon [9, 11]. Plasmids pNXB1 and pNXB3 constructed on the basis of plasmid pNXB were used for insertion of the *flgB1*- or *flgB3* gene into the chromosome of strain Δ*flgA*Δ*flgB*. To obtain the plasmid pNXB, the sequence of B-operon with flanking regions, total size of 2850 bp, in plasmid pNX was cloned by the sites of restriction endonucleases *Hind*III and *Xba*I. Owing to the fact that the nucleotide sequences of *H. salinarum* B-flagellin genes practically coincide through approximately 200 bp from the 5'-end and 100 bp from the 3'-end, the gene sequences show identical sites of restriction endonucleases. Two restriction endonucleases, *Mva*I and *Van*91I, were selected for restriction of *B*-flagellin genes in the 5'-terminal and 3'-terminal regions, respectively. At the same time, neither of these enzymes restricts the sequence of plasmid pNX. Restriction of the pNXB plasmid in the 5'– or 3'–homologous regions of *B*-flagellin genes by restrictases *Mva*I and *Van*91I, respectively, followed by ligation of the major fragment of the plasmid to itself, was used to obtain new plasmids pNXB1 and pNXB3 bearing either *flgB1* or *flgB3* surrounded by the flanking regions of the *B* operon. The ligase reaction was carried out overnight at 16°C in the manufacturer's with the recommended vector, insert, and ligase quantities. Obtaining and transformation of competent *E. coli*

cells and isolation of plasmid DNA were performed as in [16].

Deletions and insertions of the flagellin genes were obtained by the following procedure (exemplified by obtaining strain Δ*flgA*Δ*flgB* from strain Δ*flgA* with the plasmid pNXΔB): Δ*flgA* cells were transformed by plasmid pNXΔB and inoculated to an agarized medium containing novobiocin and X-Gal. Since it is not a replication plasmid, the transformed cells could grow in such conditions only after incorporation of the plasmid into the chromosome by the mechanism of homologous recombination. Accordingly, inclusion of the plasmid into the chromosome could occur only by the flanking regions of the *B* operon, in a random way, either on the right or on the left of the *B* operon. The blue coloration of all colonies (resulting from the enzymatic reaction of the halophilic β-galactosidase) was an additional marker confirming plasmid incorporation. After the colonies had become blue (day 14 of growth), they were reinoculated to a large volume (200 ml) of culture medium without the antibiotic and grown for a further five days. Growth without selective pressure could result in a repeated act of homologous recombination accompanied by plasmid exclusion. Since the plasmid carried two regions homologous to the chromosome (to the left and right of *B* operon), plasmid exclusion was equiprobable by any of these regions. If the plasmid was excluded by the region opposite to the one used for incorporation, then the chromosomal fragment containing the *B* operon was deleted. After cultivation in the medium without novobiocin, the cells were inoculated once again on the agarized medium without the antibiotic in the presence of X-Gal. After 14 days, colorless colonies (the plasmid in their cells was absent) appeared on this medium, about 1% of the total number of colonies. The bulk was made up by blue-colored colonies (with the plasmid retained in the chromosome). Among the colorless colonies, those with the genome reconstituted to the genome of wild type cells (plasmid exclusion without deletion) were present, as well as deletion mutants (plasmid exclusion with the capture of the *flgB* operon). Mutant strains with the chromosomal DNA bearing only the *flgB1*- or *flgB3* gene were obtained using plasmids pNXB1 and pNXB3, respectively. The inserts of these genes into the chromosome of strain Δ*flgA*Δ*flgB* were obtained similarly to the method of "deletion in frame," with the difference that the successive inclusion–exclusion of the plasmid resulted not in deletion but in insertion of the necessary sequences. The search for deletion mutants and control of the inserts of individual *flgB* genes was accomplished using PCR and Southern blot hybridization according to [16].

Flagellar filaments were isolated from cell cultures by precipitation with polyethylene glycol as described previously [9]. Protein electrophoresis in PAAG in the presence of SDS was carried out in accordance with [17]. Immunoblotting was performed with ProtoBlot Western Blot Ap (Promega) according to the manufacturer's recommendations. Antisera were obtained by immunization of rabbits with purified flagella as in [18]. Mass spectra of the tryptic fragments of proteins under study were obtained on a MALDI-TOF-MS Bruker mass spectrometer (Germany) at the Department of Proteomic Research, Orekhovich Institute of Biomedical Chemistry, Russian Academy of Medical Sciences.

Electron microscopy was performed on a JEM-100c microscope (JEOL, Japan). The samples were placed on formvar-coated electron-microscopic copper grids and contrasted with 2% uranyl acetate. For immunoelectron microscopy, filament preparations were exposed according to [19] with polyclonal antibodies against wild-type flagella of *H. salinarum* and conjugates of protein A with colloid gold particles (10 nm) (Jannsen Biotech, Olen, Belgium).

RESULTS AND DISCUSSION

Previously, our team obtained strains *H. salinarum* Δ*flgA* and Δ*flgB* [9, 11]. In this work, we have obtained strain *ΔflgAΔflgB* lacking *A* and *B* flagellin genes by the introduction of additional deletion of *B* operon into the chromosome of strain Δ*flgA*. Strain *ΔflgAΔflgB* does not differ from the wild-type strain in growth rate, but has no motility, according to the light microscopy analysis of cell behavior. Since only A and B flagellins had been found previously as components of the flagellar filaments of wild-type cells, we expected that the filamentous structures would completely disappear after deletion of the encoding genes. However, electron microscopy showed that *ΔflgAΔflgB* cells had short filamentous structures which we designated as X-filaments. In contrast to the wild type cells, which for the most part had bundles of several spiral flagella, few cells of the mutant strain had single X-filaments localized over the whole cell surface. X-filaments (7–8 nm) are approximately 1.5 times thinner than the normal flagella (11–12 nm) and, in contrast to the wild type flagella and the mutant filament forms observed previously, have no marked characteristic shape (Fig. 1b, 4a). Structures similar to X-filaments have not been found previously in wild type cells.

Here, the question arises as to the composition, origin, and function of X-filaments. It would be logical to suppose that these filaments are built of the sixth flagellin protein XXX. We have made an attempt to isolate and purify these structures using the method of flagella isolation [9]. A preparation was obtained which is a mixture of numerous proteins according to the results of electrophoresis in PAAG (Fig. 2). This result may be due to the extremely low quantity of X-filaments and concurrent precipitation of other subcellular structures. Obviously, the minor protein of X-filaments escaped detection in the presence of other flagellins. On the other hand, other proteins coprecipitated with X-filaments possibly also participated in the formation of more complex supramolecular structures. The prepara-

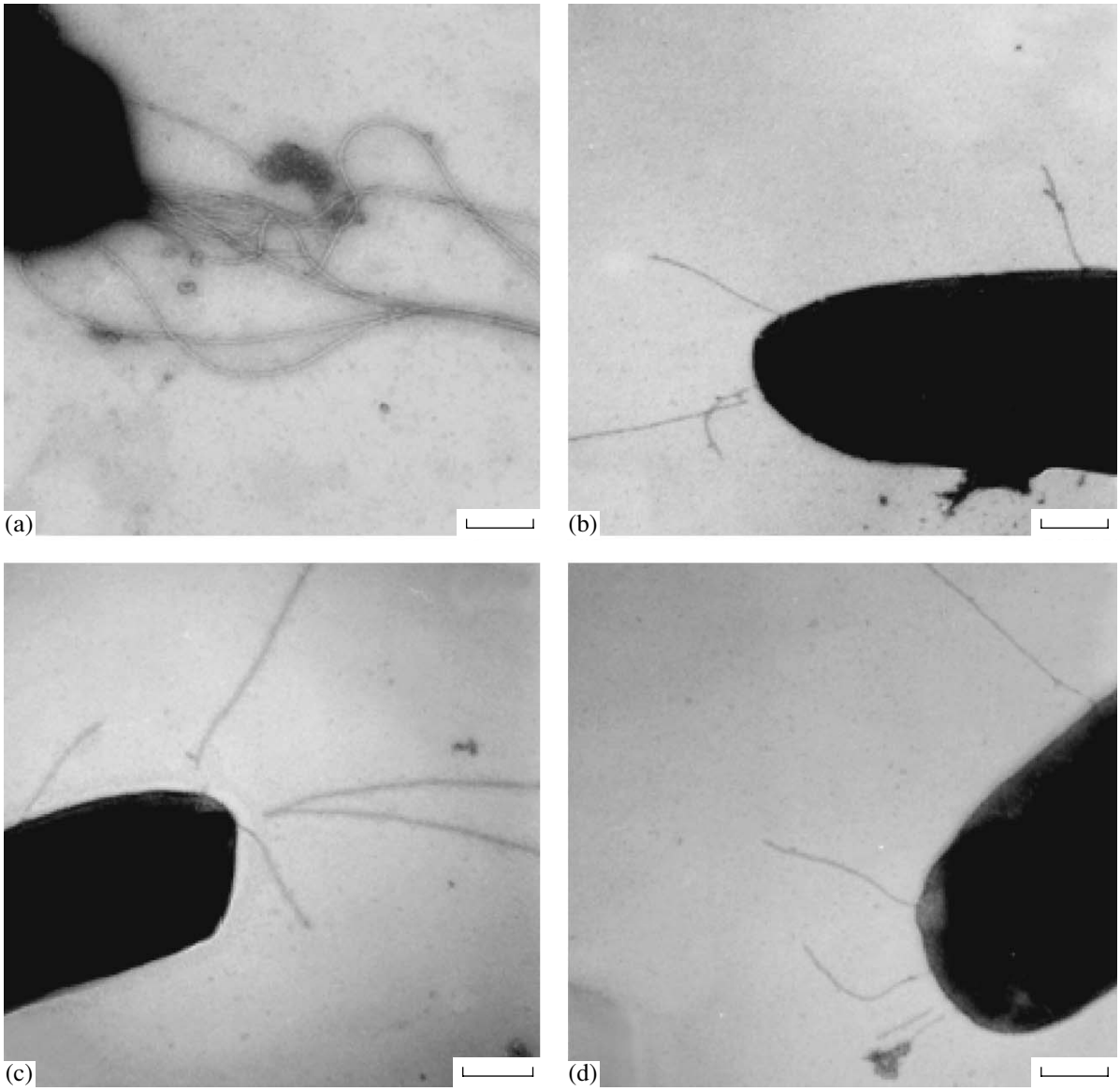

Fig. 1. Electron-microscopic photographs of wild-type *H. salinarum* cells (a) and mutant strains: Δ*flgA*Δ*flgB* (b), Δ*flgA*Δ*flgB2*Δ*flgB3* (c), and Δ*flgA*Δ*flgB1*Δ*flgB2* (d), negatively stained with 2% uranyl acetate. Scale bar: 0.3 mkm.

tion of X-filaments after electrophoresis was analyzed by immunoblotting (Fig. 3). As can be seen from this figure, the "lower" band shows high affinity to specific antibodies against wild-type flagella, whereas the "upper" band is less markedly labeled with antibodies. Direct evidence for the specific affinity of X-filaments to antibodies against wild-type flagella was given by immunoelectron microscopy (Fig. 4). X-filaments are most probably assembled from the protein comprising the "lower" band. We have made an attempt to identify this protein by mass spectrometry of tryptic fragments. However, the masses of the obtained peptides did not correspond to any of those predicted for *H. salinarum* proteins. It should be noted that the team of Oesterhelt in Germany also encountered difficulties in the identification of the proteins of the *H. salinarum* motility apparatus by mass spectrometric analysis [20, 21]. Nevertheless, to date these researchers have successfully identified, with different degrees of reliability, all proteins of the *fla* locus and flagellins of the *A*- and *B* loci comprising the cell, except for the *flgXXX* gene product [12].

We assume that X-filaments are assembled of the *flgXXX* gene product, because the observed properties of the protein forming them correspond to those expected for XXX-flagellin. Identification of XXX-flagellin was possibly prevented by posttranslational modifications, the nature of which may be similar to modifications described for archaeal flagellins and bacterial pilins [6]. We expect that additional deletion of

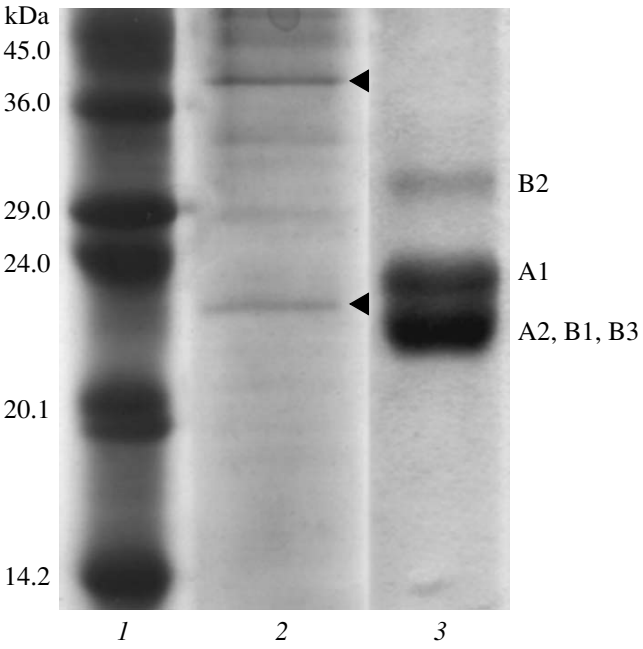

Fig. 2. Electrophoresis in denaturing gel: protein markers (*1*), preparations of X-filaments (*2*) and wild-type flagella (*3*) obtained after precipitation with polyethylene glycol. In the preparation of X-filaments, the "upper" and "lower" bands are indicated by triangles.

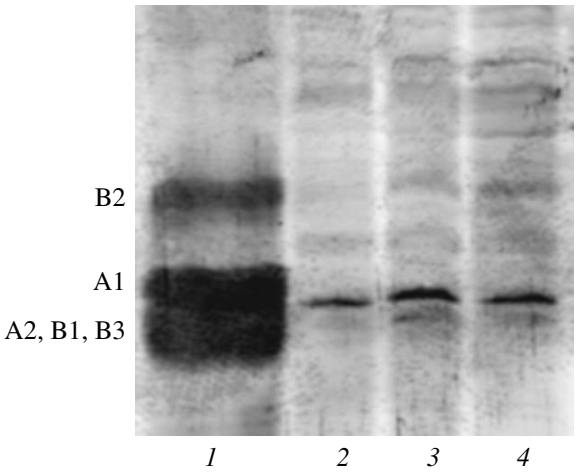

Fig. 3. Immunoblotting of *H. salinarum* filament preparations obtained by PEG precipitation: *1*, wild type; *2*, strain Δ*flgA*Δ*flgB*-, *3*, strain Δ*flgA*Δ*flgB2*Δ*flgB3*-, and *4*, strain Δ*flgA*Δ*flgB1*Δ*flgB2*. Polyclonal rabbit antibodies against purified wild type flagella were used.

the *flgXXX* gene in strain *ΔflgAΔflgB* will result in the disappearance of X-filaments. We have already started working in this direction.

In the framework of studying the role of *B*-flagellins, we have obtained novel mutant strains Δ*flgA*Δ*flgB2*Δ*flgB3* (with the *B1* flagellin gene only) and Δ*flgA*Δ*flgB1*Δ*flgB2* (with the *B3* flagellin gene only) on the basis of strain Δ*flgA*Δ*flg*B through insertion of the *flaB1* and *flaB3* genes by plasmids pNXB1 and pNXB3, respectively. Since the *flaB2* gene is enclosed by highly homologous genes from both sides, the obtaining of the mutant strain Δ*flgA*Δ*flgB1*Δ*flgB3* (with the *flaB2* gene only) is an extremely difficult technical problem and requires nonstandard gene engineering techniques.

Strains Δ*flgA*Δ*flgB2*Δ*flgB3* and Δ*flgA*Δ*flgB1*Δ*flgB2* also had no motility and did not differ from the wild type strain in growth rate. These strains were analyzed by electron microscopy (Fig. 1c, 1d). Both of them did not differ from strain Δ*flgA*Δ*flgB* in the shape and quantity of detected filaments. The preparations of filaments from these strains obtained by standard methods of flagella isolation were also analyzed using immunoblotting (Fig. 3, lanes *3–4*). As is seen, no additional bands corresponding to *B1* or *B3* flagellin were observed.

The results obtained by our team previously show interdependence between the synthesis and assembly of A and B flagellins [9]. Thus, the deletion of the *B* locus leads to a significant decrease of the expression of *A* genes as compared with the wild type. Although the filaments synthesized in the case of *B* locus deletion are similar to the native ones in length and shape, the cells of the mutant strain are devoid of motility. Apparently, the presence of *B*-flagellins is necessary for the correct attachment of filaments to the rotating motor axis and, despite a considerable similarity of amino acid sequences, A-flagellins cannot replace *B*-flagellins at this stage of assembly. Deletion of the *A* locus and even the *flgA2* gene alone reduces the quantity of matrix RNA for the *B* operon to a non-detectable level. At the same time, as we have shown previously [9, 11], the strain with the deletion of the *A*-flagellin operon has short curved structures which resemble in appearance the flagellar hooks described for bacteria and a number of methanogenic archaea and are localized over the whole cell surface. Only *B2*-flagellin was found in the flagella preparation of this strain.

The introduction of single *B1* or *B3* flagellins into strain Δ*flgA*Δ*flgB* did not result in the formation of curved flagellar hook structures. Hence, other *B* proteins are unable to undertake the key role in the formation of hook-like filaments in the absence of *B2* flagellin, and *B2* flagellin is their only major component. The question is still open whether additional minor proteins, besides the major *B2* flagellin, are required for the formation of hooks. Besides *B1* and *B3*, XXX-flagellin and some *fla* locus proteins may act as such proteins. Furthermore, it seems possible that there are adjacent structures between the spiral filament of the flagellum and the hook, as well as between the hook and the membrane motor components, like those forming hook-associated proteins (HAP) in bacteria [22]. Since *B1* and *B3* flagellins are components of wild-type flagella and their genes are successively encoded in a single *B43*-flagellin locus, the following scheme of incorporation of *B*-flagellins into the growing flagellar filament seems to be logical: after the formation of the spiral

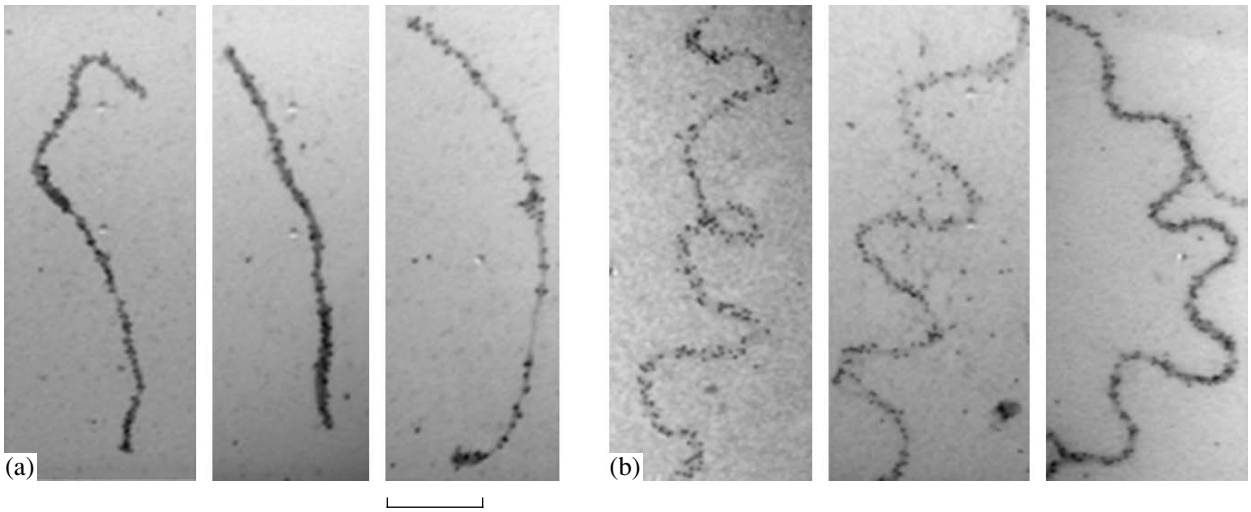

Fig. 4. Electron-microscopic photographs of X-filaments (a) and normal flagella (b) labeled with antibodies against wild type flagella (using protein A conjugates with colloid gold particles (10 nm)). Negative staining with 2% uranyl acetate. Scale rule: 0.2 μm.

flagellar filament of *A*-flagellins, *B1*-flagellin forms a short region for attachment of the hook assembled of *B2* flagellin. At the next stage, a region built of *B3* flagellin is added to the hook for direct attachment to the rotary structures in the membrane motor.

According to the modern concepts, the multicomponent structure of archaeal flagella is their distinctive feature, necessary for the formation of the flagellar filament spiral structure [7, 9–11]. In the work on flagellum formation in methanogenic archaeon *Methanococcus voltae* carrying four flagellin genes (*flaA, flaB1, B2*, and *B3*) it has been shown that the morphologically pronounced curved hook consists of *B3*-flagellin alone [10]. *B1* and *B2*-flagellins are the main components of the filament, while the minor *A*-flagellin is distributed along the filament and its function is still unclear. It should be noted that protein *B3* of *M. voltae* is not a flagellin in the strict sense and must be referred to as a hook protein. The amino acid sequence of *B3* critically differs from the highly homologous *B1* and *B2*. Close homologs of *B3* have been found in the genomes of some related methanogenic archaea (*Methanococcoides jannaschii, Methanococcus maripaludis*, and *Methanococcus vannielii*), and it may be expected that the products of the corresponding genes are also hook proteins. Genomes of other archaea (including *H. salinarum*) have no apparent close homologues of the respective gene among the flagellin genes. It should also be mentioned that the electron microscopic studies of both cells and flagella of *H. salinarum* (wild type) revealed no regions morphologically different from the filament and resembling the hooks of methanogens. In terms of general considerations, the hook is an obligatory element of a flagellum [23]. It may be assumed that the morphological differences of the hook and filament regions of *H. salinarum* can become apparent in full measure only in rotating flagella.

Our works have shown for the first time that a structure resembling a flagellar hook is formed in *H. salinarum* in the absence of *A*-flagellins. This structure is formed by one major protein: *B2*-flagellin. However, as in the case of *M. voltae*, we cannot refer to *B2* protein as a hook protein in a strict sense. It is not improbable that there is a more complex interaction between the *flgA*, *flgB* and *flgXXX* gene products in the case of expression of *A*-flagellins. Probably, the role of *B*-flagellins will be defined more exactly when the mutant strains are obtained in which different *flgB* genes will be incorporated into the region of deleted *A* operon, since the regulatory sequences of *A* operon permit noticeable synthesis of *A* proteins continuing even after *B* operon deletion.

The recent study of the flagella of the hyperthermophilic archaeon *Pyrococcus furiosus* [24] gives evidence that its flagella are "multifunctional organelles" and, besides providing cell motility in liquid medium, are responsible for adhesion to different surfaces and formation of intercellular cablelike structures, which may play an important role during the transfer of genetic material at cell–cell contacts. It is probable that *H. salinarum* flagella can also perform similar functions; at the same time, flagellin proteins can differ in the degree of their involvement in such functions. It may be supposed that the role of a protein forming X-filaments has to increase significantly in the case of deletion of the *flgA* and *flgB* genes, which leads to the interruption of the synthesis of full-fledged flagella. Furthermore, presuming the involvement of respective organelles in cell–cell interaction with the wild type cells, theoretically one may anticipate the recovery of damaged flagellin loci as a result of genetic recombination of DNA.

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