Archaeal type IV pre pilin-like signal peptidases
Szabo, Zalan

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CHAPTER 4

FLAGELLAR MOTILITY AND STRUCTURE IN THE HYPERTHERMOACIDOPHILIC ARCHAEON SULFOLOBUS SOLFATARICUS

Zalán Szabó, Musa Sani, Maarten Groeneveld, Benham Zolghadr, James Schelert, Sonja-Verena Albers, Paul Blum, Egbert J. Boekema and Arnold J. M. Driessen

Submitted for publication

Abstract

Archaeal flagella are, like those of bacteria, surface appendages required for swimming motility. However, the mode of secretion and assembly of archaeal flagella is more similar to that of bacterial type IV pili. The structural subunits, flagellins and pilins, contain a characteristic type IV pilin-like (class III) signal sequence, and several components of the secretion/assembly apparatus of these two systems are related. Here we have investigated flagellation and motility in the hyperthermoacidophilic crenarchaeon Sulfolobus solfataricus. Unlike other archaea, the flagellum of Sulfolobus ssp. consists of only a single structural protein FlaB. The flaB gene was found to be strongly induced at nutrient-limiting conditions. The flaJ accessory gene was inactivated by targeted gene disruption and the resulting mutant lacked flagella on the cell surface and was found non-motile. Finally, processing of electron microscopy images of isolated flagella reveals its architecture as a right-handed 3-start helix with a 54 Å pitch.
**Introduction**

The ability of organisms to actively change location can greatly increase their chances of survival in an ever-changing environment. Bacteria achieve this by numerous ways, of which the best studied examples are twitching motility mediated by retractable type IV pili, and swimming motility driven by rotating flagella (23). In the Archaea, the second domain of prokaryotic life, an intriguing merger of the two bacterial systems is observed. Archaea are a diverse group of organisms often found in extreme environments with high temperatures, high salinity, or high or low pH. Many archaea possess flagella that serve swimming motility. However, the structural subunits (flagellins) are assembled by a mechanism that resembles the biogenesis of the type IV pilus. Bacterial flagella are hollow tubes assembled by a highly complex type III secretion machinery (125). These structures are assembled at the tip which requires the transport of the flagellar subunits from the base to the tip through the hollow tube. Archaeal homologs of this system have not been identified. Instead, homologs of type IV pilus biogenesis proteins can be found in archaeal genomes and archaeal flagellins. Such structures typically assemble at the base. The archaeal flagellins contain a highly conserved N-terminal type IV pilin-like signal sequence. Type IV pilin-like signal sequences resemble secretory signal peptides as they contain a short positively charged leader peptide followed by a hydrophobic (H-) domain of about 20 amino acids. After insertion into the membrane, however, only the short N-terminal leader peptide is cleaved off at the cytosolic face of the membrane by a dedicated signal peptidase. The mature protein still contains the hydrophobic part (13,20,205) and this domain plays a crucial role in the subsequent assembly of pilin or flagellin subunits at the base of the growing filament (44). Three components of archaeal flagellar biogenesis are related to proteins involved in type IV pilus biogenesis: the prefailin peptidase [FlaK in *Methanococcus maripaludis* (20) and PibD in *Sulfolobus solfataricus* (13)], the type II/IV secretion system ATPase homolog FlaI, and the polytopic membrane protein FlaJ. FlaJ contains a conserved GspII_F PFAM-domain (26) and bacterial proteins bearing this domain are thought to serve as an assembly platform for type IV (pseudo)pili. These accessory genes have been shown to be required for flagellum biogenesis in *Methanococcus voltae* (21,216). Additionally, a *Halobacterium salinarum* flaI mutant lacked flagella and was deficient in motility on semi-solid agar plates (162). In analogy with the type IV pilus/type II secretion systems, FlaI and FlaJ are assumed to constitute the core of the machinery that assembles flagella (14,163).

Structural studies on the flagellar filament from *H. salinarum* provided additional evidence for the relatedness of archaeal flagella and type IV pili. Both are thin helical filaments (6-9 nm and approximately 10 nm in diameter, respectively) with a central hydrophobic core which is most likely comprised by the conserved N-terminal α-helix that includes the H-domain of the signal sequence (41,44,217). Swimming motility has so far been demonstrated for several euryarchaea and the crenarchaeon *Sulfolobus acidocaldarius*, and for *H. salinarum* and *Methanococcus voltae* it was shown to be dependent on the flagellar filaments comprised of the structural FlaB proteins (2,31,88,117,129,203).
Flagellation in *Sulfolobus solfataricus*

So far, flagellation has been studied in molecular detail in model organisms belonging to the *Euryarchaeota*. However, little is known about flagellar structure and motility in the *Crenarchaeae*, the second main archaeal phylum (64,70,117). This report describes studies on the flagellar system of the thermoacidophilic crenarchaeon *Sulfolobus solfataricus*. Strong transcriptional induction of the structural *flaB* gene was observed under certain growth conditions. A mutant deficient in the flagellar accessory gene *flaJ* lacked flagella and was non-motile. Furthermore, we present a structural model for the flagellar filament based on single particle electron microscopy and suggest that the C-terminal part of flagellin may contribute to the oligomerization of the flagellar protein.

**Materials and Methods**

*Strains and growth conditions.* *Escherichia coli* DH5α was used for all cloning steps. For the production of protein, an *E. coli* strain C43(DE3)/pACYC-RIL ((135) and Stratagene) was employed. *Sulfolobus solfataricus* P2 (obtained from the Deutsche Sammlung von Mikroorganismen und Zellkultur GmbH in Braunschweig, Germany) and *S. solfataricus* PBL2025 (187) and the *flaJ::lacS* disruption strain were grown aerobically at 80°C and pH 3 in Brock’s basal salts medium (minimal medium, MM) (35). This medium was supplemented with either 0.2% trypton (Difco), 0.1% yeast extract and 0.2% sucrose (referred to as “rich medium” in the text), or 0.2% of sugar (glucose or arabinose).

Due to the unusual growth requirements of *Sulfolobus* species (80°C and pH 3), special care must be taken when growing these strains on solid media. Traditionally, gelrite at a concentration of 0.6% (w/v) is used in combination with Ca²⁺ and Mg²⁺ salts as a solidifying agent rather than agar (189). A range of gelrite concentrations was tested to obtain a semi-solid medium, and the optimal concentration was determined to be 0.1%.

To prepare semi-solid medium, 0.2% gelrite (Serva, Heidelberg, Germany), dissolved by stirring in boiling demineralized water, was added to an equal volume of 2 x concentrated Brock’s minimal medium containing 0.02% glucose, and 20 mM magnesium chloride and 6 mM calcium chloride to solidify the medium. The pH of the medium was adjusted to 3. To inoculate the plates, cells grown on Brock’s medium supplemented with 0.1% glucose were pelleted (10 min, 3500 x g, 21°C) and resuspended in 0.1 volume of culture supernatant. Then, a 10 µl droplet of this suspension was applied to the center of the plate and allowed to absorb into the medium. Plates were incubated for 5-6 days in a sealed humid chamber at 80°C.

*Plasmids and primers.* The plasmids used in this study are listed in Table 1. To obtain recombinant cleaved FlaB, a plasmid was constructed that allows for simultaneous expression of substrate (*flaB*) and processing peptidase (*pibD*) genes in *E. coli*. A cassette containing a 6xHIS tagged *pibD* under control of the T7 promoter that could be inserted into the unique SphI restriction site in pZA8 was constructed. First, pZA5 was cut with SalI and the purified 6732 bp fragment religated, to eliminate an SphI restriction site downstream of the *pibD* coding region. From the resulting plasmid a 2503 bp EcoRV fragment was ligated into the unique HindII restriction site of pUC18 (146). From this construct a 1538 bp SphI fragment was excised, which contained a T7 promoter followed by the *pibD* coding region, a carboxy-terminal 6xHIS tag and a T7 terminator sequence. This fragment was inserted into the single SphI restriction site of pZ28, yielding pZ28- *pibD*. Truncated versions of *flaB* were constructed as follows. To obtain a *flaB* variant lacking the coding region of amino acids 1-156, plasmid pZA1 DNA was cut with NcoI and NdeI, filled in with Klenow fragment (Roche, Almere, Netherlands) and religated, yielding pMG1. A truncate lacking the coding region for amino acids 1 -54 was obtained by PCR amplification of a bp fragment using primers 5’-CCCATCGAGGAGGAGGAGCAGCGTAAC-3’ and 5’- CCGGATCCTATTACTGATACGCTACC-3’ (NcoI and BamHI restriction sites underlined) and pZA1 as a template. The PCR product was digested with NcoI and BamHI and ligated into the corresponding restriction sites of pSA4, resulting in pMG2. For targeted disruption of the *flaJ* gene,
plasmid pET2275 was generated. A lacS cassette containing the gene with its own promoter and terminator region amplified using primers 5'- CCCCCCATGGCTCTCTTATTAGAATTGTACGC-3' and 5'- CCCCCGGATCCCTAGTGTTGCAAGGCAG-3' (NcoI and BamHI restriction sites underlined) with *S. solfataricus* P2 genomic DNA as a template. was ligated into the pET401 cloning vector (K.H.M. van Wely, unpublished results) cut with NcoI and BamHI yielding pET2268 and its endogenous EcoRV site was removed by site directed mutagenesis (240). The N-terminal (673 bp) and C-terminal (728 bp) coding region of flaJ were amplified by PCR using primers 5'- CCCCCGTACCAGCGAATAAGGAAATGAGCAG-3' and 5'- CCCCCGTACCAGCGAATAAGGAAATGAGCAG-3' (KpnI and NcoI restriction sites underlined), 5'- CCCCCGGATCCAGGTATAGATTGTATGAAAAT-3' and 5'- CCCCCCGCGGCCGCTTAAATAAAGCCGTGGATGTCG-3' (BamHI and NotI restriction sites underlined), respectively, using *S. solfataricus* P2 genomic DNA as a template. The cut PCR products were inserted into pET2268 using the appropriate restriction enzymes resulting in pET2275. The flaJ disruption strain was constructed as described in Worthington et al. (240).

**Table 1. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD/Myc-His A</td>
<td><em>E. coli</em> expression vector, arabinose inducible promoter</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSA4</td>
<td>pET15b derivative, T7 promoter, C-terminal 6×HIS tag in frame with a BamHI restriction site</td>
<td>(13)</td>
</tr>
<tr>
<td>pZA1</td>
<td>pSA4-flaB-6×HIS</td>
<td>(13)</td>
</tr>
<tr>
<td>pZA5</td>
<td>pSA4-pibD-6×HIS</td>
<td>(13)</td>
</tr>
<tr>
<td>pZA8</td>
<td>pBAD/MycHisA-flaB-HA</td>
<td>(205)</td>
</tr>
<tr>
<td>pZA8-pibD</td>
<td>pBAD-flaB-HA/T7-pibD-6×HIS</td>
<td>This study</td>
</tr>
<tr>
<td>pMG1</td>
<td>pZA1-flaB[Δ1-156]-6×HIS</td>
<td>This study</td>
</tr>
<tr>
<td>pMG2</td>
<td>pSA4-flaB[Δ1-54]-6×HIS</td>
<td>This study</td>
</tr>
<tr>
<td>pET401</td>
<td>pBluescript SK+ derivative, engineered NcoI site</td>
<td>K.H. van Wely (unpublished), (226)</td>
</tr>
<tr>
<td>pET2268</td>
<td>pET401-lacS cassette</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Total RNA isolation and Northern analysis.** RNA was isolated from *S. solfataricus* P2 cells as described (6). For analysis of flaB gene transcript levels, 5 µg of total RNA was separated on a denaturing 1.1% agarose gel and subsequently capillary blotted onto Zeta-probe membranes (BioRad). Non-radioactive hybridization and detection was performed as described (108), using a flaB specific probe.

**Protein overproduction, cell fractionation, protein enrichment.** FlaB precursor was expressed as described (13). To obtain cleaved FlaB, cells carrying pZA8-pibD were grown until an OD<sub>600</sub> of 0.6-0.8 and L-arabinose was added to a concentration of 0.2% to induce FlaB production. The culture was shaken for 2 h at 37°C and subsequently pibD expression was induced by the addition of 0.1 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) for 2 h. Cells were harvested by centrifugation and resuspended in a buffer containing 50 mM Tris/HCl, 10 mM EDTA, pH 7.5. Crude membranes from these cells were isolated as described (13). To produce N-terminally truncated FlaB versions, pMG1 or pMG2 were transformed into *E. coli* strain C43(DE3)/pACYC-RIL and bacteria were grown until an OD<sub>600</sub> of 0.6-0.8. Then, 0.5 mM IPTG was added and the culture was incubated for 4 h at 37°C. Cells were then harvested by centrifugation, resuspended in buffer containing 50 mM Tris/HCl and 10 mM EDTA, pH 7.5 and lysed by sonication on ice (MSE Soniprep 150; 8 cycles of 15 sec at an amplitude of 8 µm, with 45 sec pauses). Lysed material was centrifuged (10 min, 8,000 x g, 4C). The pellet was washed three times in phosphate buffered saline containing 1% (v/v) Triton X-100 to remove cellular material, and resuspended in 50 mM sodium phosphate buffer, pH 8.0. This sample was designated insoluble protein fraction. The supernatant of the low speed centrifugation was fractionated into membrane and soluble fractions by ultracentrifugation at 267,000 x g. The membrane pellet was resuspended in 50 mM sodium phosphate buffer, pH 8.0. At each step, pellets were resuspended in volumes identical to the supernatant fractions.
Isolation of flagella from culture supernatants. To obtain a crude flagellar preparation, an *S. solfataricus* PBL2025 culture was grown in Brock’s minimal medium supplemented with 0.1% glucose until stationary phase. Then, cells were removed by centrifugation (30 min, 3,000 x g). The supernatant was re-centrifuged to remove residual cells. The resulting culture supernatant was pelleted by ultracentrifugation (20 min, 26,000 x g, 4°C). This pellet contained numerous flagella as well as cellular debris and was used for initial electron microscopy observations. A final centrifugation step (220,000 x g) resulted in a pellet which mainly consisted of flagella, as observed by electron microscopy. This material was used for detailed analysis of flagellar ultrastructure.

Electron microscopy and Single-Particle Analysis. For image processing, a flagellar filament suspension was negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. For determination of the handedness of the helical packing of the filaments, metal shadowing was performed on carbon/formvar – coated grids. Unstained flagella were air-dried and rotary shadowed with platinum. Electron microscopy was performed on a Philips CM120 electron microscope operating at 120 kV with a LaB6 filament. Images were recorded with a 4000 SP 4K slow-scan CCD camera at 80,000 x magnification at a pixel size of 3.75 Å at the specimen level with "GRACE" software (152). Single particle analysis (62) was performed with the Groningen Image Processing ("GRIP") software package on a PC cluster. Slightly overlapping segments were extracted from the micrographs. To correct for the significant in-plane curvature, we first rotationally aligned all segments. The best 60% of the rotationally oriented segments, as judged by using the correlation coefficient as the quality criterion, were then further processed by full alignment procedures. The aligned projections were treated with multivariate statistical analysis in combination with hierarchical classification (227) before final averaging.

Results

Analysis of the *fla* gene locus in *Sulfolobus solfataricus*. The *S. solfataricus* P2 flagellum operon is disrupted by an insertion sequence (IS element) integrated into the coding region of the *flaG* gene (SSO2321 (192)). In a related strain, *S. solfataricus* PBL2025 (see below) the IS element is absent as shown by PCR amplification and sequence analysis of the relevant gene region (data not shown). The coding sequence was identical in both *S. solfataricus* strains. The reconstructed flagellum (*fla*) operon encodes seven open reading frames (Figure 1A): one structural protein (suffix B), five putative accessory proteins (G, F, H, I, J) and one ORF (SSO2322) of which homologs can only be found in the other two available *Sulfolobus* genomes (38,100). In *Sulfolobus*, only one flagellin gene can be identified, while all other archaea analyzed so far contain multiple flagellin genes (24). While FlaB and products of the *flaHIJ* gene cluster are readily identified by sequence similarity searches, the *flaG* and *flaF* gene products are less well conserved (Table 2). Although SSO2322 does not show homology to any known flagellar accessory protein, it contains a predicted coiled coil region which might be important for protein-protein interactions. To find conserved regions within the *flaB* promoter, sequences upstream of *flaB* genes obtained form the three available *Sulfolobus* ssp. genome sequences were aligned using ClustalW (39). A five base pair direct repeat was detected, starting 75 bases upstream of the translational start site and 30 bases upstream of a putative TATA box and BRE site. In conclusion, for all but one gene products of the *S. solfataricus* *fla* operon a possible function could be assigned based on sequence homology.
Transcriptional regulation of the **flaB** gene. We aimed to find conditions under which the flagellin gene is induced, assuming that under these conditions cells are also producing flagella. To do so, Northern blot analysis on RNA isolated from cells grown under different conditions was performed using part of the **flaB** gene as a probe. First, early and late growth phases were compared (Figure 1B). While very little amounts of gene transcript were detected in samples from the mid-logarithmic growth phase, a strong induction in stationary cells was observed. This induction might be due to limitation of nutrient availability, or a possible bacterial-type quorum sensing mechanism (50). No homologs of bacterial quorum sensing systems have been detected in archaea so far (34). To determine whether nutrient limitation induces **flaB** expression, cells were shifted from rich to minimal medium and samples were taken for Northern analysis. A cell culture grown in rich medium to mid-logarithmic phase was collected by centrifugation and resuspended in either rich medium, or minimal medium (MM) without addition of a carbon source. Samples were taken before, and one and two hours after the shift and analyzed as described above (Figure 1C). No increase in **flaB** transcript levels was observed when cell s continued to grow on rich medium. However, a strong induction could be detected after two hours incubation in MM. Up-regulation of **flaB** was confirmed by semi-quantitative RT-PCR performed on RNA isolated from cells grown under the conditions described above (not shown). In addition, by semi-quantitative RT-PCR, a moderate **flaB** induction in cells grown on sugars (glucose or arabinose) as compared to peptides (tryptone) could be detected. In summary, the flagellin gene seems to be preferentially expressed in cells that are exposed to unfavorable nutritional conditions.
Flagellation in *Sulfolobus solfataricus*

Figure 1. Genomic organization of the *S. solfataricus* fla operon and transcriptional regulation of flaB. (A). Schematic representation of the *S. solfataricus* fla operon. Genes are shown as open arrows with gene designations based on homology. The structural flagellin gene is in black, genes with predicted accessory function are grey. Suffixes of fla genes with identified homologies are depicted in the boxes while locus tag (SSO) numbers are given above. SSO2322 (white arrow) is unique to the genus *Sulfolobus*. A conserved 5 base direct repeat (underlined), starting 75 bases upstream of the translational start site of flaB, is shown. The consensus sequence was derived by aligning flaB promoter sequences from three *Sulfolobus* species. Nucleotide base codes are: Y = C or T, N=any nucleotide. (B) Northern analysis of the flaB gene transcript after transfer of a cell culture from rich into minimal medium. RNA was isolated from cells before, and one and two hours after the shift into either rich medium (TYS) or basal salts medium (MM). The flaB gene transcript was detected using a specific DIG-labeled probe (top panel). The positions of the 16S and 23S ribosomal RNAs, determined by methylene blue staining of the blot membrane (lower panel), are indicated. (C) Detection of flaB gene transcript levels at different growth stages (top panel). RNA was isolated from cells grown in rich medium to mid logarithmic (L) or stationary (S) phase. Identical amounts of RNA to those used in the Northern blot were separated on 1.5% agarose gels and stained with ethidium bromide (lower panel).

Construction of a flaJ disruption mutant. To determine whether the flaJ accessory gene is involved in flagellar biogenesis and motility in *S. solfataricus*, a disruption mutant was constructed in the lactose auxotrophic strain PBL2025. *S. solfataricus* PBL2025 contains a large chromosomal deletion which also includes lacS, the gene coding for β-glycosidase and accordingly, these cells are not able to grow on lactose as the sole carbon source (187). The flaJ gene was targeted using a suicide plasmid (pET2275) carrying the flaJ coding region interrupted at base 685 by the lacS gene as a selectable marker (Figure 2A). After electroporation of PBL2025 with pET2275, a strain carrying the integrant was selected on liquid minimal medium supplemented with lactose and purified as described before (240). The integrity of the mutant was confirmed by PCR amplification of part of the flaJ gene region (Figure 2B). As expected, using DNA from the wild-type strain as a template and primers
Figure 2. Construction of a flaJ disruption mutant. (A) Schematic representation of the genomic environment of the flaJ gene and the suicide plasmid used to target the gene. Arrowheads indicate primers flaJ1 and flaJ2. Recognition sites of selected restriction enzymes on the genomic fragment are HindIII (H), EcoRI (E) and ScaI (S). (B) PCR on genomic DNA isolated from strain PBL2025-flaJ::lacS or strain PBL2025 (wt).

flaJ1 and flaJ2, a 428 base pair fragment was amplified (Figure 2A and B). A PCR performed on DNA isolated from the flaJ::lacS strain with the same primers resulted in a larger fragment of 2.4 kb corresponding to the flaJ gene carrying the lacS insert (Figure 2B). Also, the two regions overlapping the flaJ flanks and the selection marker were amplified by PCR and analyzed by direct sequencing of the PCR products. The resulting flaJ::lacS mutant strain and the wild-type strain were characterized for motility and the presence of flagella. Cells grown in liquid glucose medium were negatively stained with 2% uranyl acetate and examined by transmission electron microscopy (Figure 3A and B). Cells of the wild-type strain were peritrichous, without any bundling of filaments. Longer filaments of up to several micrometers in length were also frequently observed. On the other hand, cells of the flaJ strain completely lacked flagella on the cell surface (Figure 3B). To establish whether flagella conferred motility in *S. solfataricus*, a swarming assay on semi-solid plates was established. Minimal medium plates containing 0.05% glucose and 0.1% gelrite as the solidifying agent (see materials and methods), were inoculated with a droplet of approximately $10^7$ cells. Plates were incubated for 6 days at 80°C in a sealed humid chamber. Strain PBL2025 formed a dense circular spot of cells corresponding to the area of inoculation, and a lighter halo around this region, consistent with a swarming phenotype (Figure 3C). The formation of chemotactic rings (183) was not observed, consistent with previous observations of the swimming behavior of *S. acidocaldarius* cells (117). In contrast, flaJ cells did not swarm on semi-solid gelrite plates (Figure 3D). Therefore, the flaJ gene product is required for the biogenesis of flagella on *S. solfataricus* cells as well as for swarming motility.
Figure 3. Phenotypic characterization of PBL2025 and flaJ strains. (A) and (C) transmission electron microscopy images of negatively stained cells. (B) and (D) Motility was assayed on semi-solid gelrite plates supplemented with MM + 0.05% glucose. Scale bar is 200 nm for (A) and (B).

**Structural features of isolated flagella.** *Sulfolobus* species carry only one copy of the flagellin gene and do not show reversed swimming or tumbling (13,70,117,192). Therefore, we argued that the structure of flagella from this organism should be particularly homogenous, in contrast to polymorphic flagella from *Halobacterium salinarum* that is built up of multiple subunits (217). To obtain structural information about the flagellar filament, series of electron micrographs was collected from negatively stained specimens isolated from culture supernatants. Long filaments of up to several micrometers extending out of the grid plane were frequently observed (Figure 4A). Segments of filaments which were rather straight were extracted from micrographs for image analysis (Figure 4B). A single particle approach was used for processing. A final average projection map of 120 segments is shown in Figure 4D which clearly shows that the filament has a helical packing. The diameter of the filament is 145 Å and the pitch (repeat between the helices; indicated by white lines in Figure 4D) is 54 Å. However, the filament is not composed of a single strand, but has a three-stranded helical arrangement, as indicated in the scheme of Figure 4D. Unfortunately, although the resolution of the two dimensional map is about 18 Å, the repeating protein motif in the strands is not resolved. No attempt was therefore undertaken to reconstruct the three dimensional shape of the filaments. Surface metal shadowing was performed to retrieve information about the handedness of the expected helical packing of the filaments (Figure 4C). It can be seen that the contribution of the helices in one direction, from the lower-left to upper-right, is stronger than in the upper-left to lower-right direction. This indicated that the filaments are composed of right-handed helices (Figure 4C).
Figure 4. Electron microscopy analysis of the *S. solfataricus* flagellum. (A) Example of a long flagellar filament showing a typical wave-like pattern. (B) A typical segment of a flagellum, used for image processing; the helical motif of the filament is readily recognizable (C) Projection average of 33 aligned metal shadowed segments indicates that the filament is right-handed. (D) Two dimensional projection average of 120 aligned segments. (E) Scheme for the helical packing projected on the averaged image of frame (D). The 3-start left-handed helical packing is indicated in shades of gray. The scale bar is 1 µm for frame (A) and 10 nm for frames (D) and (E). See Appendix 2 (page 117) for a colour version of this figure.

*In vitro* analysis of heterologously expressed FlaB variants. Bacterial type IV pilins have a strong tendency to aggregate but become soluble when the N-terminal α-helical portion is removed (46,75,101). Recombinant *S. solfataricus* full-length flagellin precursor and mature variants showed a weak tendency to form putative dimers as determined by SDS-PAGE and Western immunoblot analysis (Figure 5A). Surprisingly, two FlaB truncates lacking parts of the N-terminal portion (54 and 156 amino acids, respectively) were mostly recovered from the insoluble pellet fraction after overexpression in *E. coli* (Figure 5B). Additionally, a strong band at about 45 kDa was observed for FlaBΔ[1-54], corresponding to a putative dimer of the protein. In the case of FlaBΔ[1-156] a ladder-like distribution of bands was observed, indicating the possible formation of higher-order oligomers. These additional bands were still present when low-speed pellet (P-) fractions were pre-incubated at temperatures up to 55°C in SDS sample buffer immediately prior to loading samples on the gel (Figure 5C). When samples were pre-incubated at 90°C, FlaBΔ[1-54] migrated as a single band and the ladder-like pattern formed by FlaBΔ[1-156] mostly disappeared. To determine whether these aggregates were formed by interaction of only FlaB truncate molecules, 6xHIS-tagged FlaBΔ[1-54], solubilized from the insoluble fraction (see Materials and methods), was purified by immobilized metal affinity chromatography. The purified protein migrated as multiple bands in the gel (Figure 5D) that could be disassembled by heating in SDS sample buffer. This suggests that the protein has an intrinsic capacity to form homo-oligomers.
Flagellation in *Sulfolobus solfataricus*

**Figure 5. Migration pattern in SDS-PAGE of heterologously expressed full-length and truncated flagellin.** FlaB was detected with anti-6xHIS tag-antibody. (A) Full-length precursor and mature FlaB (preFlaB/FlaB) are mostly monomeric with a minor fraction migrating at the molecular weight of a putative dimer (arrow). (B) Sub-cellular localization of truncated FlaB variants expressed in *E. coli*. S, soluble fraction; P, insoluble low-spin pellet fraction; M, membrane fraction. (C) Stability of FlaB(Δ[1-54]) after pre-incubation in SDS sample buffer at various temperatures, immediately prior to sample loading. (D) Aggregation behavior of purified FlaB(Δ[1-54]), with or without heating the sample.

**Discussion**

*Sulfolobus* species inhabit hot acidic volcanic environments that are typically low in carbon sources. A number of strategies for efficient survival in this environment have evolved, including a membrane which is particularly impermeable to protons at high temperatures (222) and a dynamic heat shock response (206). Another important factor is the flagella dependent motility of these organisms. *Sulfolobus acidocaldarius* cells swim straight while regular pauses allow for changes in swimming direction by Brownian motion, a type of motility referred to as random walk. Based on simulations, it was suggested that despite the absence of a thermotactic behavior, a population of *S. acidocaldarius* can migrate from a higher to a lower temperature in the presence of a sharp thermal gradient due to temperature dependent variations of swimming speed (117). In this way, cells benefit from being motile while avoiding lethal hot spots in their environment. Although a bacterial-like chemotaxis system is found in many archaeal genomes, it has not been detected in *S. solfataricus* (192). This suggests that if motility is regulated, it occurs at a more global level. Our data suggest that flagellation is controlled at the level of transcription. The finding that the flaB gene is strongly induced when cells are depleted of carbon source is substantiated by earlier observation that cells are motile under such conditions (117). Regulation of flagellin genes was also observed in the methane producing archaeon *Methanocaldococcus jannaschii* which uses molecular hydrogen as an energy source (142). Induction of flagellar protein synthesis was detected at high cell densities and excess hydrogen, or at low hydrogen partial pressure. Furthermore, a 10 base direct repeat with a three base spacing was identified upstream of the flaB1 gene of *M. jannaschii*, which could be a transcriptional regulator binding site (215). From an alignment of the flaB promoter nucleotide sequences from the three sequenced *Sulfolobus* species a conserved five base direct repeat, separated by a six base spacer, can be deduced.
This sequence is 30 base pairs upstream of the putative TATA-box and BRE sites and is a candidate binding site for a transcriptional regulator. Thus, in \textit{M. jannaschii} and \textit{S. solfataricus}, flagellation is induced under comparable conditions and regulation seems to occur at the level of transcription. Future studies will need to address the molecular details of this regulation.

The \textit{fla} operon of \textit{Sulfolobus} species contains fewer genes than any other flagellated archaeon (Figure 1). In this way, it can be regarded as a minimal motility system. An intriguing question is whether the proteins encoded by this gene cluster comprise the full flagellar apparatus, namely assembly machinery as well as motor and membrane anchoring structures. Recent technical advances in the genetic manipulation of \textit{S. solfataricus} make this organism an interesting model to further investigate the mechanism of flagellar assembly and function in molecular detail (10,77,187).

Flagellation as well as motility was confirmed to be dependent on an accessory protein homolog by targeted disruption of the \textit{flaJ} gene (Figures 2 and 3). This is in line with published evidence for \textit{H. salinarum} and \textit{M. voltae} (162,216), although an effect of the mutation on motility was not assayed in \textit{M. voltae}. FlaJ is the only polytopic membrane protein in the \textit{fla} operon and based on its similarity to bacterial GspF proteins, it is tempting to speculate that it constitutes the platform on which the flagellum is assembled in the cytoplasmic membrane, and possibly the attachment site for the ATPase FlaI (6). Interestingly, FlaJ proteins contain two GspII\_F domains which may be due to an internal duplication event of the original \textit{flaJ} gene (163).

The availability of a non-flagellated \textit{S. solfataricus} mutant will also facilitate the identification and analysis of other surface appendages in this organism. For example, attachment of \textit{Sulfolobus} cells to granular sulphur has been observed and shown to be dependent on thin pili with a thickness of about 5 nm (235). Using the \textit{flaJ} mutant, it will now be possible to observe and isolate these thin filaments in the absence of the dominating flagellar structures. Also, other types of surface exposed organelles have been proposed to exist in \textit{S. solfataricus} (6,14), Szabo \textit{et al.}, unpublished).

Archaeal flagellins and bacterial type IV pilins are thought to follow similar assembly paths (24). The elucidation of the exact macromolecular architecture of type IV pilis is one of the current challenges in the field (44). This is hampered by the thin diameter and smooth surface of type IV pilis which makes EM analysis extremely difficult (44). Archaeal flagella are thicker in diameter and the subunits have a higher molecular weight, resulting in a structure that is more suitable for obtaining medium resolution structures, as shown for \textit{H. salinarum} (41,217), and in this study. Because \textit{S. solfataricus} flagella are composed of only one type of flagellin (64), we expected that its electron microscopy structure would be more homogenous than flagella from other archaea which are composed of multiple flagellins (213). Our image processing strategy was to treat segments of the flagellar filament as single particles to produce average a two dimensional projection map, thereby increasing the quality and resolution of the image. The final assembly results in a channel-less tube of a right-handed, three start helically packed proteins with a constant diameter of 145 Å and a pitch of 54 Å. The absence of a channel precludes a flagella-like monomer assembly and supports a pilus like assembly. A three dimensional model of the \textit{S. solfataricus} flagellum at higher resolution than the 18 Å in negative stain is necessary to make a better comparison to polymorphic flagella from \textit{Halobacterium salinarum} that is built up
of multiple subunits. In this context, it will be important to obtain a crystal structure of single flagellin subunits, or flagellin head domains.

Our in vitro studies with recombinant FlaB variants indicate that while full-length *S. solfataricus* flagellin has a certain tendency to form dimers, truncation of the conserved N-terminal hydrophobic domain results in the spontaneous formation of stable oligomers. Possibly, removal of the putative N-terminal transmembrane segment resulted in a conformational change by which a domain required for protein-protein interaction was exposed, leading to extensive aggregation of the protein. In the in vivo situation, flagellin subunits might undergo a conformational change during their assembly into the flagellum, leading to stronger interaction between the C-terminal head domains and stabilization of the flagellar filament. This might be a strategy to produce flagella that withstand the extreme environmental conditions in which *Sulfolobus* cells survive while uncontrolled oligomerization of FlaB in the cytoplasmic membrane is avoided. Archaeal flagella are, in contrast to bacterial type IV pili, not retractable and therefore the polymerization of flagellin subunits is most likely an irreversible process. As a consequence, extensive interactions between flagellin subunits are possible. Since purified FlaB[Δ1-54] also formed oligomers, it appears that the observed protein-protein interactions are specific and significant to assembly and function of flagella from this hyperthermophilic organism.

In this study we have investigated flagellation in *S. solfataricus* and established a basis for future investigations of cell surface exposed structures. Because flagella are abundant on *Sulfolobus* cells, the availability of a non-flagellated mutant will significantly ease the investigation of other types of membrane bound organelles, including the bindosome, a putative assembly of ABC transporter binding proteins involved in the efficient uptake of various sugars (6,14)

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