Haloferax volcanii cells lacking the flagellin FlgA2 are hypermotile

Manuela Tripepi,^{1†} Rianne N. Esquivel,^{1†} Reinhard Wirth² and Mechthild Pohlschröder¹

Correspondence

Mechthild Pohlschröder
pohlschr@sas.upenn.edu

¹Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA

²Institute for Microbiology, University of Regensburg, Universitaetsstrasse 31D-93053 Regensburg, Germany

Motility driven by rotational movement of flagella allows bacteria and archaea to seek favourable conditions and escape toxic ones. However, archaeal flagella share structural similarities with bacterial type IV pili rather than bacterial flagella. The *Haloferax volcanii* genome contains two flagellin genes, *flgA1* and *flgA2*. While FlgA1 has been shown to be a major flagellin, the function of FlgA2 is elusive. In this study, it was determined that although FlgA2 by itself does not confer motility to non-motile $\Delta flgA1$ *Hfx. volcanii*, a subset of these mutant cells contains a flagellum. Consistent with FlgA2 being assembled into functional flagella, FlgA1 expressed from a plasmid can only complement a $\Delta flgA1$ strain when co-expressed with chromosomal or plasmid-encoded FlgA2. Surprisingly, a mutant strain lacking FlgA2, but expressing chromosomally encoded FlgA1, is hypermotile, a phenotype that is accompanied by an increased number of flagella per cell, as well as an increased flagellum length. Site-directed mutagenesis resulting in early translational termination of *flgA2* suggests that the hypermotility of the $\Delta flgA2$ strain is not due to transcriptional regulation. This, and the fact that plasmid-encoded FlgA2 expression in a $\Delta flgA2$ strain does not reduce its hypermotility, suggests a possible regulatory role for FlgA2 that depends on the relative abundance of FlgA1. Taken together, our results indicate that FlgA2 plays both structural and regulatory roles in *Hfx. volcanii* flagella-dependent motility. Future studies will build upon the data presented here to elucidate the significance of the hypermotility of this $\Delta flgA2$ mutant, and will illuminate the regulation and function of archaeal flagella.

Received 21 May 2013

Accepted 22 August 2013

INTRODUCTION

The flagellum, which is the only archaeal motility structure that has been identified thus far, is one of the most intriguing and well-studied structures found in archaea (Lassak *et al.*, 2012b; Ng *et al.*, 2008). As shown for *Halobacterium salinarum*, flagella form a helical bundle and allow swimming by means of rotational movement (Alam & Oesterhelt, 1984; Marwan *et al.*, 1991). However, although bacterial and archaeal flagella have similar functions, archaeal flagellar rotation is driven by ATP (Streif *et al.*, 2008), which is consistent with the recent identification of the putative *Sulfolobus acidocaldarius* ATPase motor, a fundamentally different process from the ion-gradient-driven bacterial motor (Berg, 2003; Ghosh *et al.*, 2011; Reindl *et al.*, 2013).

Moreover, there is no homology between the structural components of bacterial and archaeal flagella nor between

the components involved in the assembly process, which led to the proposal to rename the archaeal flagellum as ‘archaellum’ (Jarrell & Albers, 2012). We will continue using the term flagellum as the scientific community has not yet come to a consensus on this term (Wirth, 2012).

In particular, while these rotating motility structures are assembled using some archaea-specific components, archaeal flagella also share homology with components involved in the biosynthesis of bacterial type IV pili (Ghosh & Albers, 2011; Jarrell & McBride, 2008; Pohlschröder *et al.*, 2011; Thomas *et al.*, 2001). Like type IV pilins, the archaeal flagellins are derived from precursors that have an amino-terminal signal peptide that targets the flagellins to the Sec pathway, which transports these precursor proteins laterally inserting the hydrophobic domain into the cytoplasmic membrane. At the membrane, a type IV prepilin peptidase homologue (FlaK/PibD) removes the charged N-terminal region of the signal peptide and the mature protein is incorporated into a flagellar structure (Albers *et al.*, 2003; Bardy & Jarrell, 2002, 2003). A hydrophobic domain at the amino-terminus of the mature flagellin constitutes the core of the structure (Albers &

[†]These authors contributed equally to this paper.

Abbreviation: 5-FOA, 5-fluoroorotic acid; CsCl, caesium chloride.

A supplementary table and two videos are available with the online version of this paper.

Pohlschröder, 2009; Cohen-Krausz & Trachtenberg, 2008; Trachtenberg & Cohen-Krausz, 2006).

Archaeal flagella lack the inner channel that is characteristic of bacterial flagella, where the flagellins are incorporated at the tip of the flagellum after passing through the channel. In archaea, flagellins are believed to be incorporated at the bottom of the flagellar structure in a process that involves FlaI and FlaJ, which are homologous to type IV pilus biosynthesis components PilB, an ATPase, and PilC, a membrane protein, respectively (Banerjee *et al.*, 2012; Chaban *et al.*, 2007; Lassak *et al.*, 2012b). This difference is reflected in the sizes of these two flagellar structures; archaeal flagella are thinner than bacterial flagella (10–14 nm compared with 18–22 nm) (Jarrell & Koval, 1989; Jones & Aizawa, 1991), but generally have larger diameters than type IV pili (5–9 nm) (Jarrell & Albers, 2012).

Like type IV pili, which generally contain major pilins that are the primary structural component of the bacterial type IV pilus, and several minor pilin-like proteins that play important roles in the regulation, assembly and/or function of the pilus (Craig *et al.*, 2006; Giltner *et al.*, 2012), most archaea contain several flagellins. The flagellum of the haloarchaeon *Hbt. salinarum*, for example, encodes five flagellins that share homology with each other (Beznosov *et al.*, 2007; Tarasov *et al.*, 2000). Microscopy studies revealed that flagellin A1 alone or flagellins A1 and A2 expressed in the absence of the B1–B3 flagellins are able to form polar flagella similar to the WT, while the flagella encoded by *flgB1*–*B3* are shorter than the WT flagella and showed surface structures that resembled sacs filled with basal body-like structures. These flagella were also distributed over the whole cell surface. However, all three mutant backgrounds resulted in cells with a severe motility defect and their roles in flagella biosynthesis and function are still elusive (Beznosov *et al.*, 2007; Tarasov *et al.*, 2000).

Methanococcus voltae expresses four flagellins, two proposed major flagellins, FlaB1 and FlaB2, as well as two minor flagellins, FlaA and FlaB3. FlaA is distributed throughout the entire flagellum and cells lacking this minor subunit are still motile but at a level below that of the WT (Bardy *et al.*, 2002; Jarrell *et al.*, 1996). Conversely, FlaB3 localizes in the proximal section of the cell, and it is possible that this subunit constitutes the hook region of the flagellum, although more data are required to confirm this (Bardy *et al.*, 2002). *Methanococcus maripaludis* has three flagellin genes *flaB1*–*B2*–*B3*. FlaB1 and FlaB2 are the major subunits that are required for flagella filament assembly and motility of the cells (Thomas & Jarrell, 2001). Interestingly, the lack of the minor flagellin FlgB3 does not result in a phenotype on plates used to assess swimming motility, but the cells swim abnormally in circles when analysed with phase-contrast microscopy (Chaban *et al.*, 2007).

The only known archaea that appear to only contain one flagellin gene include members of the Sulfolobales, including *Sulfolobus solfataricus*, *S. acidocaldarius* and

Sulfolobus islandicus (Lassak *et al.*, 2012a, b; Szabó *et al.*, 2007) and the haloarchaeon *Haloarcula lacusprofundi* (Siutkin *et al.*, 2012). The model haloarchaeon *Haloferax volcanii* genome contains two flagellin genes *flgA1* and *flgA2* that appear to be co-regulated (Tripepi *et al.*, 2010). MS analysis of caesium chloride (CsCl) density gradient fractions containing purified flagella revealed that FlgA1 is highly abundant, while FlgA2 is present in relatively low amounts. Consistent with the hypothesis that FlgA1 is a major flagellin, a *flgA1* deletion renders *Hfx. volcanii* cells non-motile (Tripepi *et al.*, 2012).

In this study, we determined that the deletion of the *Hfx. volcanii* gene encoding the flagellin FlgA2 does not inhibit motility. On the contrary, cells harbouring this deletion are hypermotile. Our characterization of the Δ *flgA2* strain indicates that FlgA2 may have both structural and regulatory roles in *Hfx. volcanii* flagella biosynthesis.

METHODS

Reagents. All enzymes used for standard molecular biology procedures were purchased from New England BioLabs, except for iProof High-Fidelity DNA polymerase, which was purchased from Bio-Rad. The ECL Plus Western blotting detection system and horseradish peroxidase-linked sheep anti-mouse antibodies were purchased from Amersham Biosciences. The PVDF membrane, MF membrane filters (0.025 µm) and Ultracel-3K membrane were purchased from Millipore. DNA and plasmid purification kits and anti-His antibodies were purchased from Qiagen. NuPAGE gels, buffers and reagents, were purchased from Invitrogen. Difco agar and Bacto yeast extract were purchased from Becton, Dickinson. Peptone was purchased from Oxoid. 5-fluoroorotic acid (5-FOA) was purchased from Toronto Research Biochemicals. All other chemicals and reagents were purchased from either Fisher or Sigma.

Strains and growth conditions. The plasmids and strains used in this study are listed in Table 1. Unless otherwise noted, all *Hfx. volcanii* strains were grown at 45 °C in liquid or solid semi-defined modified growth medium (MGM) or casamino acids (CA) medium (Dyall-Smith, 2004). Solid medium plates contained 1.5% agar; motility plates contained 0.3% agar. To ensure equal agar concentration in all plates, agar was completely dissolved in the medium prior to autoclaving, and autoclaved medium was stirred before plates were poured. *Hfx. volcanii* strain H53 (Allers *et al.*, 2004) was grown in CA medium supplemented with tryptophan and uracil (50 µg ml⁻¹ final concentration) or in MGM without supplements. 5-FOA was added to a final concentration of 150 µg ml⁻¹ in CA medium (uracil was also added at 20% of its normal concentration, i.e. 10 µg ml⁻¹ final concentration during 5-FOA selection) for the selection of the Δ *flgA2* (Hvo_1212) deletion mutant and *flgA2*Stop replacement (see below). Strains transformed with pTA963 were grown in CA medium supplemented with tryptophan. *Escherichia coli* strains were grown at 37 °C in NZCYM medium, supplemented with ampicillin (200 µg ml⁻¹) as needed (Blattner *et al.*, 1977).

Generation of a chromosomal deletion. Chromosomal deletions were generated using a homologous recombination (pop-in pop-out) method, as described by Allers & Ngo (2003). Plasmid constructs for use in the pop-in pop-out knockout process were generated using a modified version of the overlap PCR method described by Hammelmann & Soppa (2008). In brief, approximately 700 nt of the upstream and downstream flanking regions of the genes of

Table 1. Plasmids and strains

Plasmid/strain	Relevant properties	Reference
Plasmids		
pTA131	Amp ^r , pBluescript II with <i>Bam</i> HI– <i>Xba</i> I fragment from pGB70 containing <i>pdfx-pyrE2</i>	Allers <i>et al.</i> (2004)
pTA963	Amp ^r , <i>pyrE2</i> and <i>hdrB</i> markers, inducible <i>ptna</i> promoter	Allers <i>et al.</i> (2010)
pUC19	Amp ^r	Yanisch-Perron <i>et al.</i> (1985)
pMT9	pTA963 containing <i>flgA1His</i>	Tripepi <i>et al.</i> (2012)
pJY2	pTA131 containing chromosomal flanking regions of <i>flgA2</i>	This study
pMT21	pTA963 containing <i>flgA1flgA2His</i>	This study
pMT10	pTA963 containing <i>flgA2His</i>	This study
pMT26	pUC19 containing <i>flgA2His</i>	This study
pMT27	pUC19 containing <i>flgA2StopHis</i>	This study
pMT28	pT131 containing <i>flgA2StopHis</i>	This study
Strains		
DH5 α	<i>E. coli</i> F [−] ϕ 80lacZ Δ M15 (<i>lacZYA</i> – <i>argF</i>)U169 <i>recA1 endA1 hsdR17</i> (<i>r_K[−] m_K⁺</i>) <i>phoA supE44 thi-1 gyrA96 relA1</i>	Invitrogen
DL739	<i>E. coli</i> MC4100 <i>recA dam-13::Tn9</i>	Blyn <i>et al.</i> (1990)
H53	<i>Hfx. volcanii</i> Δ <i>pyrE2</i> , Δ <i>trp</i>	Allers <i>et al.</i> (2004)
MT2	H53 Δ <i>flgA1flgA2</i>	Tripepi <i>et al.</i> (2010)
MT13	H53 containing pTA963	Tripepi <i>et al.</i> (2012)
MT40	H53 containing pMT9	This study
MT41	H53 containing pMT21	This study
MT42	H53 containing pMT10	This study
MT14	H53 Δ <i>flgA1</i>	Tripepi <i>et al.</i> (2012)
MT15	MT14 containing pTA963	Tripepi <i>et al.</i> (2012)
MT16	MT14 containing pMT9	Tripepi <i>et al.</i> (2012)
MT54	MT14 containing pMT10	This study
MT90	MT14 containing pMT21	This study
MT30	H53 Δ <i>flgA2</i>	This study
MT43	MT30 containing pTA963	This study
MT89	MT30 containing pMT9	This study
MT56	MT30 containing pMT10	This study
MT55	MT30 containing pMT21	This study
MT44	MT2 containing pTA963	This study
MT46	MT2 containing pMT9	This study
MT47	MT2 containing pMT10	This study
MT45	MT2 containing pMT21	This study
RE26	H53 Δ <i>pilB3C3</i>	R. Esquivel, unpublished results

interest were amplified using overlap and restriction primers (see Table S1, available in *Microbiology* Online, for a list of primers). More specifically, the upstream and downstream flanking regions were amplified independently of each other, each containing a restriction site and an overlap fusion site. The products of this initial PCR (the upstream and downstream regions) were then mixed together in equal concentrations and amplified for several cycles without the addition of primers. Temperatures for these cycles were as follows: 98 °C for 30 s, six cycles of 98 °C for 10 s, ~50 °C for 30 s and 72 °C for 1 min, and a 72 °C hold. The primers were then added at 72 °C and the PCR continued for 30 more cycles at 98 °C for 10 s, ~60 °C for 30 s and 72 °C for 1 min. This second fusion PCR produces the fused upstream and downstream flanking regions with the appropriate restriction sites for cloning into the haloarchaeal suicide vector pTA131. The restriction sites used specifically for the flagellin knockouts were *Bam*HI and *Xba*I.

Motility assay. Motility assays were performed on plates containing 0.3 % agar in MGM or CA medium, supplemented with tryptophan

or uracil depending on the strain being assayed. A toothpick was used to stab inoculate the agar, and halo sizes around the inoculation site were measured.

Construction of expression vectors encoding proteins with carboxy-terminal His tags. Plasmids containing gene fusions encoding flagellins that have a carboxy-terminal His tag were created. These expression vectors included the inducible tryptophanase promoter (*tna*) to drive expression of these gene fusions. The genes encoding FlgA1His and FlgA2His were cloned in a manner similar to that used for the co-expression of FlgA1 and FlgA2His {FlgA[1–2]His}, as previously described (Tripepi *et al.*, 2012). Primers used specifically for the construction of pMT10 and pMT21 (pTA963 expressing carboxy-terminally His-tagged FlgA2 and FlgA[1–2], respectively) are listed in Table S1. pMT10 and pMT21 were isolated from *E. coli* DH5 α and used to transform *E. coli* DL739 (Table 1). Using a standard PEG (Dyall-Smith, 2008), non-methylated plasmid DNA isolated from *E. coli* DL739 was used to transform *Hfx. volcanii* strain H53.

Insertion of stop codon into *flgA2*. To express a chromosomally encoded *flgA2* containing a stop codon at position 135, (*flgA2Stop*), we performed the following steps [the QuikChange site-directed mutagenesis protocol (Stratagene) was used with modifications]. The gene encoding FlgA2His was excised from pMT10 and cloned into pUC19 (Yanisch-Perron *et al.*, 1985). The plasmid was then amplified using a set of oligonucleotide primers containing a mutation that resulted in the replacement of the codon for glutamate at position 135 with a stop codon (see Table S1). PCR amplification of the pUC19 insert containing the sequence encoding the FlgA2His fragment was performed using Phusion high-fidelity DNA polymerase, which has a non-strand-displacing action that allows incorporation of the mutagenic primers, resulting in a nicked circular strand. The methylated, non-mutated parental DNA template was digested by using the restriction enzyme *DpnI*, and the remaining mutated DNA was then transformed into *E. coli* DH5 α . Plasmid DNA was isolated from transformed cells and sequenced to test for the presence of the point mutations. The mutated gene was excised and cloned into the pTA131 plasmid. We used the same strategy (pop-in pop-out) employed in making the deletion mutant but selected for the recombination that resulted in *flgA2Stop* insertion into the chromosome. The successful insertion was verified by PCR and by DNA sequencing.

RNA purification. Total RNA was isolated from *Hfx. volcanii* cells (at exponential phase; OD₆₀₀ 0.4–0.6) using RNeasy RNA purification columns (Qiagen). RNA was treated with amplification-grade DNase I according to the recommendations of the supplier (Sigma-Aldrich). The integrity of RNA was determined by agarose gel electrophoresis. SuperScript II reverse transcriptase (Life Technologies) was used for cDNA synthesis, and amplifications of the gene of interest were performed using the primers listed in Table S1.

Protein extraction, lithium dodecyl sulfate-PAGE and Western blotting. Liquid cultures were grown to exponential phase (OD₆₀₀ ~0.5). Cells were collected by centrifugation at 4300 *g* for 10 min at 4 °C. Cell pellets were resuspended and lysed in 1 % NuPAGE lithium dodecyl sulfate supplemented with 50 mM DTT. Samples were loaded onto Bis-Tris NuPAGE gels (Invitrogen) under denaturing conditions using MOPS at pH 7.7, and proteins were separated by electrophoresis. Proteins were transferred from the gel onto a PVDF membrane using a Bio-Rad Transblot-SD semidry transfer cell at 15 V for 30 min. Western blots of whole-cell lysates of *Hfx. volcanii* strains expressing His-tagged constructs were probed with an anti-His antibody diluted 1:1000, followed by a secondary anti-mouse antibody diluted 1:10 000. Antibody-labelled protein bands were identified using the Amersham ECL Plus Western blotting detection system.

Light microscopy. The cells analysed using light microscopy were taken from the edge of a swarm ring and the soft agar macerated in liquid MGM using a pipette tip. Ten plates were stabbed. However, the tenth plate, with cells having motility intermediate between WT and Δ *flgA2*, was not used for measurements of swimming speed. The cell mixture was placed in rolling drums at 45 °C for 2–4 h and the supernatant was analysed as previously described (Herzog & Wirth, 2012), or the macerate was subjected to a 15 s low-speed centrifugation to pellet most of the soft agar and the supernatant transferred into the rectangular glass-capillaries used for light microscopy at 45 °C. Time-compressed videos (2 frames s⁻¹, resulting in a 12-fold speed enhancement) were recorded as previously described (Herzog & Wirth, 2012) and analysed using ImageJ. The mean speed of five different cells was recorded for each experiment for both WT and Δ *flgA2*, and the final mean speed was obtained from the nine experiments.

Isolation and purification of flagella. The isolation of flagella was performed as described by Fedorov *et al.* (1994), with modifications. To select for motile cells, colonies from a solid-agar plate were stab

inoculated onto motility plates and then inoculated into 5 ml MGM liquid medium, which was further inoculated into 2 l MGM, and the cultures were harvested at OD₆₀₀ ~ 0.2 by centrifugation at 8700 r.p.m. (JA-10 rotor; Beckman) for 30 min. The supernatant was centrifuged again (8700 r.p.m. for 30 min) and incubated at room temperature with 4 % (w/v) PEG 6000 for 1 h. The PEG-precipitated proteins were then centrifuged at 16 000 r.p.m. (JLA-16.250 rotor; Beckman) for 50 min at 4 °C, and the flagella were purified by CsCl density-gradient centrifugation (overnight centrifugation at 50 000 r.p.m.) (VTI-65.1 rotor; Beckman). CsCl was dissolved in a 3 M NaCl solution to a final density of 1.37 g cm⁻³.

Electron microscopy. Samples (500 μ l) of cells were fixed in MGM with 2 % glutaraldehyde and 1 % paraformaldehyde for 1 h, and 10 μ l aliquots were placed on glow-discharged carbon grids for 10 min. The grids were rinsed twice in double-distilled H₂O and negatively stained using 1 % uranyl formate. Samples (10 μ l) of CsCl gradient density fractions were applied to copper grids coated with carbon-Formvar. The grids were left for 5 min at room temperature, washed with water, blotted with a filter paper and stained with 2 % uranyl acetate for 10 s. Grids were then analysed using a Philips Tecnai 12 instrument operating at 120 kV and a Gatan US1000 2K $\times$ 2K (2024 $\times$ 2024-pixel) camera. ImageJ software was used to measure the filament lengths of 10 filaments each for the Δ *pilB3C3* and Δ *pilB3C3* Δ *flgA2* strains.

RESULTS

Transcription of *flgA2* in a Δ *flgA1* strain

We have previously shown that an *Hfx. volcanii* Δ *flgA1* strain is non-motile (Tripepi *et al.*, 2012). We wanted to confirm that the phenotype can be directly attributed to the lack of the FlgA1-encoding region rather than being caused by a polar effect on *flgA2* transcription, whose start codon is 11 bp downstream of *flgA1* and hence likely co-transcribed with *flgA1* (Fig. 1a). We used semiquantitative reverse transcription (RT)-PCR to determine relative *flgA2* transcript levels in the parent H53 strain, referred to as the WT strain from hereon, and in the Δ *flgA1* strain. We confirmed that *flgA2* is transcribed in both strains, suggesting that the lack of FlgA1 is responsible for the motility defect (Fig. 1b).

Hfx. volcanii Δ *flgA2* and Δ *flgA2Stop* strains exhibit a hypermotile phenotype

To determine whether *Hfx. volcanii* cells can make functional flagella when expressing only FlgA1 and to further elucidate the role of FlgA2 in *Hfx. volcanii* motility, we created a Δ *flgA2* strain. We verified the deletion of *flgA2* by PCR, using primers designed to amplify the coding region or a product including the regions upstream and downstream of *flgA2*. A PCR product was obtained for the coding region for the WT, but not for the Δ *flgA2* strain (Fig. 2a). Consistent with the deletion of the 660 bp *flgA2* gene, the amplified products of a PCR using a primer pair located approximately 700 bp upstream and 700 bp downstream of *flgA2* correspond to about 2.0 and 1.4 kb when chromosomal DNA of the WT and *flgA2* deletion strain is used, respectively (Fig. 2b).

Surprisingly, when stab-inoculated on MGM or CA motility plates, this deletion strain is hypermotile (Fig.

Fig. 1. *flgA2* is transcribed in the absence of *flgA1*. Semiquantitative reverse transcription (RT)-PCR was performed on WT and $\Delta flgA1$ strains. (a) *flgA1* and *flgA2* chromosomal location. (b) *flgA2* primers were used to detect the transcript levels of this gene in the WT and $\Delta flgA1$ strains. Samples were normalized using the level of expression of the 16S rRNA gene (primers listed in Table S1).

3a, Table 2). We determined that the $\Delta flgA[1-2]$, $\Delta flgA1$, $\Delta flgA2$ and parent strains grow similarly in liquid MGM and CA medium, suggesting that this hypermotility phenotype is not caused by an increased growth rate for the $\Delta flgA2$ strain (data not shown). To the best of our knowledge, a hypermotility phenotype has not previously been reported for a flagellin-deletion mutant.

To determine whether the increase in motility is due to a transcriptional regulation of *flgA2*, we constructed a strain in which the chromosomal copy of *flgA2* was replaced with a modified *flgA2* gene in which the glutamate 135 codon (GAG) was replaced with a stop codon (TGA) (Fig. 3b). Cells expressing this gene (*flgA2Stop*) were also hypermotile, suggesting that this motility phenotype is due to a post-transcriptional effect (Fig. 3a).

With the previously described extension of our strain collection, we now have: a $\Delta flgA[1-2]$ strain (Tripepi *et al.*, 2010), lacking both flagellin genes from the start codon of *flgA1* to the stop codon of *flgA2* (including the 10 bp intergenic

region); a $\Delta flgA1$ strain (Tripepi *et al.*, 2012), lacking the *flgA1* gene from its start codon to its stop codon (but retaining the 10 bp intergenic region); and a $\Delta flgA2$ strain (see above).

Additionally, we have created three pTA963-based expression plasmids: one construct co-expresses an unmodified FlgA1 and a His-tagged FlgA2 [*flgA[1-2]His*] (Tripepi *et al.*, 2010); a second construct expresses His-tagged FlgA1 [*flgA1His*]; and the third expresses His-tagged FlgA2 [*flgA2His*]; see Methods. For all of these plasmids, the expression of the flagellins can be induced by the addition of tryptophan to the culture medium (Fig. 4).

In several cases, these deletions were introduced into a specific genetic background other than WT. For the electron microscopy analyses described below, the *flgA* deletions were introduced into a *Hfx. volcanii* $\Delta pilB3C3$ strain. PilB and PilC are part of the pilin assembly system and cells lacking these proteins do not produce type IV pilus-like structures (R. Esquivel and M. Pohlschröder, unpublished results); therefore, electron microscopy analyses of typical and atypical flagella are greatly simplified in this strain.

Complementation analysis

The non-motile phenotype of the $\Delta flgA1$ and $\Delta flgA[1-2]$ strains can be complemented by expressing *flgA1His* and *flgA[1-2]His* *in trans*, respectively (Table 2), which shows that the carboxy-terminal His-tags do not significantly affect the function of either FlgA1 or FlgA2. The incomplete rescue of the $\Delta flgA1$ strain by FlgA1His cannot be attributed solely to the carboxy-terminal His-tag since untagged versions complement only slightly better and motility does not reach WT levels in either case (data not shown).

FlgA1His expression *in trans* partially rescues the motility defect of the $\Delta flgA1$ strain and does not rescue the defect of the $\Delta flgA[1-2]$ strain, while co-expression of FlgA[1-2]His complements the motility phenotypes of both strains. Thus, it is curious that expression of FlgA1His in the WT results in a hypermotility phenotype, while the expression of FlgA2His or FlgA[1-2]His in the WT leads to increased motility but not as significant an increase in motility as that observed when FlgA1His is expressed (Table 2).

While these results indicate that the ratio of the levels of FlgA2 to FlgA1, when FlgA1 is abundant, may play a regulatory role, the complementation studies clearly show that although FlgA2 does not rescue the motility defect of a $\Delta flgA1$ strain when expressed *in trans*, it does have a functional role. This is most clearly demonstrated by the fact that FlgA1 only rescues the motility defect of a $\Delta flgA1$ strain when *flgA2* is expressed from the chromosome or when it is co-expressed with *flgA1* from a plasmid.

Given these results, the hypermotility of a $\Delta flgA2$ strain is intriguing. We analysed two configurations in which only the FlgA1 flagellin is expressed; when expressing FlgA1His in a $\Delta flgA[1-2]$ strain, no motility is observed. However, a $\Delta flgA2$ strain expressing only FlgA1 is hypermotile. This, as well as the fact that expression of FlgA2 *in trans* does not

Fig. 2. Disruption of the chromosomal *flgA2* locus. PCR amplification was performed using primers (a) specific for the *flgA2* gene, and (b) against the flanking regions located approximately 700 bp upstream and approximately 700 bp downstream of *flgA2*. The template DNA was isolated from the WT or the $\Delta flgA2$ strains.

Fig. 3. *ΔflgA2* and *ΔflgA2Stop* strains show a hypermotile phenotype. (a) WT, *ΔflgA[1–2]*, *ΔflgA1*, *ΔflgA2* strains and *flgA2Stop* (a strain carrying a codon replacement) were stab inoculated into CA motility plates, supplemented with tryptophan and uracil, and incubated at 45 °C for 3 days. All stabs and incubations were repeated at least five times for verification of the phenotype. (b) FlgA2stop amino acid sequence. The Glu135 corresponding to the codon that was replaced with a stop codon is shown in red.

complement the hypermotility phenotype, suggests that the deletion of the chromosomal *flgA2* gene might trigger a regulatory cascade that results in increased flagella production.

FlgA2 can be assembled into surface filaments

Several attempts to raise antibodies against either of the flagellins were unsuccessful. Therefore, to determine whether FlgA2 can be assembled into surface filaments, we examined cells expressing FlgA2 using electron microscopy to determine the presence or absence of flagella on the cell surface. Since we previously determined that non-flagella type IV pilus-like structures are not readily distinguishable from flagella on the *Hfx. volcanii* cell surface (Tripepi *et al.*, 2012), we expressed FlgA2 in the *ΔpilB3C3* strain that lacks type IV pilus-like structures (see above; R. Esquivel and M. Pohlschröder, unpublished results).

The *ΔpilB3C3* strain has surface filaments but when the flagellin genes are deleted in this background, the cells lack any surface filaments (Fig. 5a), strongly suggesting that the filaments observed on the cell surface of the *ΔpilB3C3* strain are exclusively flagella. No differences were detected in the motility of the WT and *ΔpilB3C3* strains (R. Esquivel and M. Pohlschröder, unpublished results), suggesting that flagella expression and structure in this strain are similar to that of the WT strain. Interestingly, the *ΔpilB3C3ΔflgA1* strain has cell-surface structures, indicating that expression of FlgA2 is sufficient for the assembly of a flagellum (Fig. 5a). However,

of approximately 100 cells of the *ΔpilB3C3ΔflgA1* strain that were analysed, only 2 had a single surface filament. Conversely, of the 130 *ΔpilB3C3* cells examined, approximately 40 % were flagellated, and generally had one or two flagella (Fig. 5a). The flagella tend to be found at the poles in the three strains that expressed filaments.

To confirm that the lack of surface-attached flagella on most *ΔpilB3C3ΔflgA1* cells was not due to FlgA2 flagella being lost during sample preparation, we determined the flagella concentration in the supernatants, using CsCl density gradient purification. Consistent with the electron microscopy examination of cell surfaces, electron microscopy examination of CsCl gradient fractions derived for *ΔpilB3C3ΔflgA1* strain culture supernatants revealed the presence of flagella, albeit at significantly lower concentration compared to the culture supernatants of the *ΔpilB3C3* strain (Fig. 5b). Interestingly, these flagella also tended to accumulate unknown aggregates that were not seen as frequently in the *ΔpilB3C3* and *ΔpilB3C3ΔflgA2* samples. In contrast, similar gradient fractions prepared for the culture supernatants of the *ΔpilB3C3ΔflgA[1–2]* strain lack any indication of flagella. These data are consistent with MS analyses of *Hfx. volcanii* WT cells, which showed that FlgA2 is only present in low concentrations in the CsCl gradient fraction containing purified flagella (Tripepi *et al.*, 2012).

Surfaces of *Hfx. volcanii* cells that lack FlgA2 have more and longer flagella than those of WT cells

After determining that *ΔflgA2* cells swim faster than the WT strain, we next wanted to know why cells that express only the major flagellin FlgA1 are hypermotile. Hypermotility has previously been observed in cells having relatively high numbers of flagella (Senesi *et al.*, 2004). Alternatively, perhaps the structure of a flagellum containing only FlgA1 is distinct from the WT flagellum consisting of FlgA1 and FlgA2 subunits. To observe flagella assembled on the surface of cells lacking FlgA2, we deleted *flgA2* in the *ΔpilB3C3* strain. The resulting strain displays the same hypermotility phenotype as the *ΔflgA2* strain (data not shown). Electron microscopy analyses of *ΔpilB3C3ΔflgA2* cells grown in liquid medium revealed that, similar to the *ΔpilB3C3* strain, about 40 % of 130 cells examined had flagella associated with them.

Table 2. *Hfx. volcanii* FlgA1- and FlgA2-dependent motility

Strains were stab-inoculated into 0.3 % agar in CA media supplemented with tryptophan and incubated at 45 °C for 3 days. Values shown are motility haloes (cm) and are the means of nine measurements (three colonies from three different plates, each). NM, non-motile strain.

Strain	pTA963	FlgA1His	FlgA2His	FlgA[1–2]His
WT	2	3	2.5	2.6
<i>ΔflgA1</i>	NM	1.5	NM	2
<i>ΔflgA2</i>	4	4	4	4
<i>ΔflgA[1–2]</i>	NM	NM	NM	2

Fig. 4. Flagellins expressed in *Hfx. volcanii* WT, and $\Delta flgA1$, $\Delta flgA2$ and $\Delta flgA[1-2]$ strains. Western blotting was performed on protein extracts from *Hfx. volcanii* WT or the mutated strains grown to exponential phase in CA medium supplemented with tryptophan and expressing either FlgA1His, FlgA2His or FlgA[1-2]His, encoded by genes expressed under the regulation of a *trp*-inducible promoter. His-tagged proteins were detected by using anti-His antibodies. Comparable amounts of protein were loaded into each lane of the gel.

However, strikingly, while the $\Delta pilB3C3$ strain generally had one or two flagella associated with single cells, the $\Delta pilB3C3\Delta flgA2$ cells most frequently contained three to five flagella per cell (Fig. 5a). Moreover, only cells lacking FlgA2 had unusually long flagella. These long filaments had mean lengths of $6.1 \pm 2.6 \mu m$ compared with $1.8 \pm 1.1 \mu m$ in the $\Delta pilB3C3$ strain. Consistent with these data, we observed significantly more flagella structures in the culture supernatant of $\Delta pilB3C3\Delta flgA2$ cells compared to that of the $\Delta pilB3C3$ strain expressing *flgA2* (Fig. 5b).

$\Delta flgA2$ cells from the swarm edge swim faster but quickly lose their ability to swim when transferred to liquid medium

The electron microscopy data suggest that the observed hypermotility of $\Delta flgA2$ cells on motility agar might be due to faster motility of the individual cells rather than a higher

percentage of motile cells compared with WT. To support this hypothesis, we used light microscopy to analyse the motility of cells taken from the edge of motility haloes and transferred to liquid culture, for both the WT and the $\Delta flgA2$ strains. Liquid cultures of both strains contained approximately 25–40 % motile cells when pre-incubated for 2–4 h in MGM in rolling drums at 45 °C (Video S1; available in *Microbiology Online*), while all cells appeared to be non-motile upon overnight incubation (data not shown). Conversely, up to 75 % of the cells were motile when cells taken from the swarm edge were macerated from soft agar and analysed immediately (Video S2). Considering that a subset of cells of all archaeal and bacterial species analysed to date readily adhere to capillary walls, and these cells appear to be non-motile when examined using light microscopy (Streif *et al.*, 2008), the percentage of motile cells at the swarm edge is likely even higher under these conditions. The overall swimming

Fig. 5. Differential expression of flagella in $\Delta flgA1$ and $\Delta flgA2$. (a) Electron micrographs of $\Delta pilB3C3$, $\Delta pilB3C3\Delta flgA[1-2]$, $\Delta pilB3C3\Delta flgA1$ and $\Delta pilB3C3\Delta flgA2$ cells fixed in 2% glutaraldehyde and 1% paraformaldehyde followed by negative staining with 1% uranyl formate. Bars, 200 nm. (b) Electron micrographs of $\Delta pilB3C3$, $\Delta pilB3C3\Delta flgA[1-2]$, $\Delta pilB3C3\Delta flgA1$ and $\Delta pilB3C3\Delta flgA2$ CsCl gradient density fractions containing flagella. The samples were negatively stained with 1% uranyl acetate. Arrows indicate filaments; bars, 0.5 μm . Inset images, magnified images of individual filaments; bars, 100 nm.

speeds varied somewhat between experiments and shorter cells appear to swim faster than longer ones, as described in Methods. Therefore, absolute numbers calculated for swimming speeds cannot be determined with a high degree of precision. However, we are confident based on the results of nine independent experiments that the cells lacking FlgA2 swim faster than the WT cells, $5.2 \pm 1.8 \mu\text{m s}^{-1}$ and $4.1 \pm 1.1 \mu\text{m s}^{-1}$, respectively.

DISCUSSION

The regulation of swimming motility plays a critical role in the adaptation and survival of many microorganisms in a variety of environments, allowing them to avoid toxic conditions and seek favourable ones. For example, haloarchaeal cells encounter environments containing broadly varied salt concentrations, requiring an ability to swim towards environments having salt concentrations closest to those that allow optimal growth.

The results presented here suggest that the flagellin FlgA2 plays a key role in regulating the flagella-dependent swimming motility of *Hfx. volcanii*. We observed that cells lacking this flagellin swim significantly faster than WT *Hfx. volcanii* cells, an unprecedented phenotype. Comparisons of cells lacking FlgA2 to WT cells using electron microscopy suggest that the faster motility of the mutant cells may be facilitated by flagella that are longer and present in larger numbers. Our results are reminiscent of those that demonstrated that the stoichiometry of minor subunits influences the length of pili (Durand *et al.*, 2005; Giltner *et al.*, 2010). For instance, in *Pseudomonas aeruginosa* cells lacking the minor pseudopilin XcpX have an increased number of pseudopili that on average are longer than those of WT cells; conversely, increased expression of this pseudopilin strongly inhibits pseudopilus biosynthesis, indicating that XcpX plays a central role in regulating the assembly of this pseudopilus (Durand *et al.*, 2005). Indeed results of protease sensitivity assays suggest that the minor subunit induces conformational changes that result in increased protease sensitivity, accelerating destabilization of the pseudopilus and possibly leading to pilus retraction. Analogously, perhaps excess FlgA2 destabilizes *Hfx. volcanii* flagella, providing a mechanism that allows a quick response to cues in environments where sessile cells are at an advantage. Such cues might be found in environments in which biofilm formation is favourable. Under these conditions, suppressed expression of the genes that encode the flagellins would not affect the number of extant flagella on the cells, making a mechanism that facilitates rapid degradation of these surface structures advantageous. Since archaeal flagella and type IV pilus-like structures are assembled by similar biosynthesis machineries, perhaps similar mechanisms regulate the disassembly of these structures as well.

However, one major difference between the XcpX and FlgA2 subunits is that, unlike XcpX, FlgA2 can be assembled into cell-associated surface filaments in cells

lacking the major subunit. In addition, expressing FlgA2 *in trans* in WT *Hfx. volcanii* leads to a slight increase in cell motility, demonstrating that FlgA2 expressed *in trans* promotes, rather than inhibits, cell motility. Moreover, although the expression of FlgA1 *in trans* does not rescue the cell motility defect of a $\Delta\text{flgA}[1-2]$ strain, its motility is restored when FlgA1 and FlgA2 are both expressed *in trans*, and FlgA1 expressed *in trans* does rescue the motility of a ΔflgA1 strain having a chromosomal copy of *flgA2* that is expressed. While the expression of *flgA1* from the chromosome is sufficient for cell motility, perhaps the inducible promoter produces relatively low levels of FlgA1, making FlgA2 essential to producing functional flagella, and indicating that these two subunits interact in forming functional structures. Perhaps, although promoting cell motility under favourable conditions, when environmental conditions do not favour cell motility, FlgA2 has a higher affinity for a cellular component other than FlgA1. For instance, it could bind to, and negatively regulate, a positive regulator of expression of the *flgA[1-2]* operon.

This would also elegantly explain why, when *flgA1* and *flgA2* are co-regulated, we observe relatively low levels of FlgA2 in the supernatant since under these 'low-level' expression conditions, most FlgA2 is sequestered through interactions with the putative positive regulator of flagellin expression. However, when cell motility is advantageous, FlgA2 might be more readily incorporated into flagella, stimulating the formation of the flagella and promoting cell motility, as well as resulting in the activation of the positive regulator of FlgA1 and FlgA2 expression.

As previously mentioned, we have been unable to raise antibodies against either flagellin, preventing us from performing immunogold-labelling studies to determine whether flagella containing both FlgA1 and FlgA2 can be assembled. Regardless of the composition of the flagella, the results of the complementation studies in which FlgA1 or FlgA2 or both are expressed *in trans* in the WT, ΔflgA1 , ΔflgA2 or $\Delta\text{flgA}[1-2]$ strains suggest that a very delicate balance of the levels of FlgA1 and FlgA2 determines cell motility. Such fine-tuning of flagella function may be particularly important for halophilic archaea, which maintain a high cytoplasmic concentration of KCl to counterbalance the osmotic stress caused by the high environmental NaCl concentrations in their habitats (Soppa *et al.*, 2008). A likely scenario may be that a rapid decrease in the salt concentration in the environment, through precipitation for example, would result in a deleterious increase in cell turgor, making cell survival dependent on the ability to quickly escape to a more suitable environment. Low-level expression of FlgA1 and FlgA2 produces few surface structures that contain predominantly FlgA1, allowing cells to respond rapidly when the sequestered FlgA2 is released and incorporated into flagella. Under this scenario, the release of FlgA2 also frees the positive regulator of flagellin expression, and *flgA[1-2]* rapidly increases, consistent with abundance of flagella and hypermotility of the ΔflgA2 strain. Apparently,

in cells lacking FlgA2, the expression level of FlgA1 determines cell motility, with cells having low levels of expression being non-motile and cells with relatively high levels being hypermotile. The factors that determine the expression levels of the flagellins and the relative amounts of each that are incorporated into the flagella are unknown. Since the genes that encode FlgA1 and FlgA2 are probably co-regulated, the levels of expression of the flagellins are expected to be similar. Along with being incorporated into the flagella, perhaps FlgA2 interacts with other membrane proteins that affect its interaction with FlgA1, through either sequestration or modification of the flagellin. Alternatively, FlgA2 may be regulated by post-translational modifications.

Many details of the function, structure and regulation of archaeal flagella remain unknown. The discoveries that certain aspects of the biosynthesis of archaeal flagella and bacterial type IV pili are very similar, as well as the identification of uniquely archaeal features of flagella biosynthesis revealed in the last few years, are very exciting and promise to lead to novel insights into the evolution of archaeal motility. This study underscores the fact that the biosynthesis of flagella and the regulation of their function are distinctly different in archaea and bacteria, but that they also differ between species of archaea. Future analyses of these distinct systems may lead to new insights into how different species adapt to a wide variety of environments.

ACKNOWLEDGEMENTS

M. P., M. T. and R. N. E. were supported by National Aeronautics and Space Administration grant NNX10AR84G. R. N. E. was also supported by National Institutes of Health cell and molecular biology training grant TM32 GM-07229. We thank Dewight Williams for advice on microscopy, and Dieter Schifferli, Jay Zhu, Fevzi Daldal, Mohd Farid Abdul Halim and Friedhelm Pfeiffer for helpful discussions.

REFERENCES

- Alam, M. & Oesterhelt, D. (1984). Morphology, function and isolation of halobacterial flagella. *J Mol Biol* **176**, 459–475.
- Albers, S. V. & Pohlschröder, M. (2009). Diversity of archaeal type IV pilin-like structures. *Extremophiles* **13**, 403–410.
- Albers, S. V., Szabó, Z. & Driessen, A. J. (2003). Archaeal homolog of bacterial type IV prepilin signal peptidases with broad substrate specificity. *J Bacteriol* **185**, 3918–3925.
- Allers, T. & Ngo, H. P. (2003). Genetic analysis of homologous recombination in Archaea: *Haloferax volcanii* as a model organism. *Biochem Soc Trans* **31**, 706–710.
- Allers, T., Ngo, H. P., Mevarech, M. & Lloyd, R. G. (2004). Development of additional selectable markers for the halophilic archaeon *Haloferax volcanii* based on the *leuB* and *trpA* genes. *Appl Environ Microbiol* **70**, 943–953.
- Allers, T., Barak, S., Liddell, S., Wardell, K. & Mevarech, M. (2010). Improved strains and plasmid vectors for conditional overexpression of His-tagged proteins in *Haloferax volcanii*. *Appl Environ Microbiol* **76**, 1759–1769.
- Banerjee, A., Ghosh, A., Mills, D. J., Kahnt, J., Vonck, J. & Albers, S. V. (2012). FlaX, a unique component of the crenarchaeal archaeellum, forms oligomeric ring-shaped structures and interacts with the motor ATPase FlaI. *J Biol Chem* **287**, 43322–43330.
- Bardy, S. L. & Jarrell, K. F. (2002). FlaK of the archaeon *Methanococcus maripaludis* possesses preflagellin peptidase activity. *FEMS Microbiol Lett* **208**, 53–59.
- Bardy, S. L. & Jarrell, K. F. (2003). Cleavage of preflagellins by an aspartic acid signal peptidase is essential for flagellation in the archaeon *Methanococcus voltae*. *Mol Microbiol* **50**, 1339–1347.
- Bardy, S. L., Mori, T., Komoriya, K., Aizawa, S. & Jarrell, K. F. (2002). Identification and localization of flagellins FlaA and FlaB3 within flagella of *Methanococcus voltae*. *J Bacteriol* **184**, 5223–5233.
- Berg, H. C. (2003). The rotary motor of bacterial flagella. *Annu Rev Biochem* **72**, 19–54.
- Beznosov, S. N., Piatibratov, M. G. & Fedorov, O. V. (2007). [Multicomponent nature of *Halobacterium salinarum* flagella]. *Mikrobiologiya* **76**, 494–501 (in Russian).
- Blattner, F. R., Williams, B. G., Blechl, A. E., Denniston-Thompson, K., Faber, H. E., Furlong, L., Grunwald, D. J., Kiefer, D. O., Moore, D. D. & other authors (1977). Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. *Science* **196**, 161–169.
- Blyn, L. B., Braaten, B. A. & Low, D. A. (1990). Regulation of pap pilin phase variation by a mechanism involving differential dam methylation states. *EMBO J* **9**, 4045–4054.
- Chaban, B., Ng, S. Y., Kanbe, M., Saltzman, I., Nimmo, G., Aizawa, S. & Jarrell, K. F. (2007). 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* **66**, 596–609.
- Cohen-Krausz, S. & Trachtenberg, S. (2008). The flagellar filament structure of the extreme acidothermophile *Sulfolobus shibatae* B12 suggests that archaeobacterial flagella have a unique and common symmetry and design. *J Mol Biol* **375**, 1113–1124.
- Craig, L., Volkmann, N., Arvai, A. S., Pique, M. E., Yeager, M., Egelman, E. H. & Tainer, J. A. (2006). Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. *Mol Cell* **23**, 651–662.
- Durand, E., Michel, G., Voulhoux, R., Kürner, J., Bernadac, A. & Filloux, A. (2005). XcpX controls biogenesis of the *Pseudomonas aeruginosa* XcpT-containing pseudopilus. *J Biol Chem* **280**, 31378–31389.
- Dyall-Smith, M. (2008). The Halo handbook: Protocols for Haloarchaeal Genetics. http://www.haloarchaea.com/resources/halo handbook/Halo handbook_2008_v7.pdf.
- Fedorov, O. V., Pyatibratov, M. G., Kostyukova, A. S., Osina, N. K. & Tarasov, V. Y. (1994). Protofilament as a structural element of flagella of haloalkalophilic archaeobacteria. *Can J Microbiol* **40**, 45–53.
- Ghosh, A. & Albers, S. V. (2011). Assembly and function of the archaeal flagellum. *Biochem Soc Trans* **39**, 64–69.
- Ghosh, A., Hartung, S., van der Does, C., Tainer, J. A. & Albers, S. V. (2011). Archaeal flagellar ATPase motor shows ATP-dependent hexameric assembly and activity stimulation by specific lipid binding. *Biochem J* **437**, 43–52.
- Giltner, C. L., Habash, M. & Burrows, L. L. (2010). *Pseudomonas aeruginosa* minor pilins are incorporated into type IV pili. *J Mol Biol* **398**, 444–461.
- Giltner, C. L., Nguyen, Y. & Burrows, L. L. (2012). Type IV pilin proteins: versatile molecular modules. *Microbiol Mol Biol Rev* **76**, 740–772.
- Hammelmann, M. & Soppa, J. (2008). Optimized generation of vectors for the construction of *Haloferax volcanii* deletion mutants. *J Microbiol Methods* **75**, 201–204.
- Herzog, B. & Wirth, R. (2012). Swimming behavior of selected species of Archaea. *Appl Environ Microbiol* **78**, 1670–1674.

- Jarrell, K. F. & Albers, S. V. (2012). The archaellum: an old motility structure with a new name. *Trends Microbiol* **20**, 307–312.
- Jarrell, K. F. & Koval, S. F. (1989). Ultrastructure and biochemistry of *Methanococcus voltae*. *Crit Rev Microbiol* **17**, 53–87.
- Jarrell, K. F. & McBride, M. J. (2008). The surprisingly diverse ways that prokaryotes move. *Nat Rev Microbiol* **6**, 466–476.
- Jarrell, K. F., Bayley, D. P., Florian, V. & Klein, A. (1996). Isolation and characterization of insertional mutations in flagellin genes in the archaeon *Methanococcus voltae*. *Mol Microbiol* **20**, 657–666.
- Jones, C. J. & Aizawa, S. (1991). The bacterial flagellum and flagellar motor: structure, assembly and function. *Adv Microb Physiol* **32**, 109–172.
- Lassak, K., Ghosh, A. & Albers, S. V. (2012a). Diversity, assembly and regulation of archaeal type IV pili-like and non-type-IV pili-like surface structures. *Res Microbiol* **163**, 630–644.
- Lassak, K., Neiner, T., Ghosh, A., Klingl, A., Wirth, R. & Albers, S. V. (2012b). Molecular analysis of the crenarchaeal flagellum. *Mol Microbiol* **83**, 110–124.
- Marwan, W., Alam, M. & Oesterhelt, D. (1991). Rotation and switching of the flagellar motor assembly in *Halobacterium halobium*. *J Bacteriol* **173**, 1971–1977.
- Ng, S. Y., Zolghadr, B., Driessen, A. J., Albers, S. V. & Jarrell, K. F. (2008). Cell surface structures of archaea. *J Bacteriol* **190**, 6039–6047.
- Pohlschroder, M., Ghosh, A., Tripepi, M. & Albers, S. V. (2011). Archaeal type IV pilus-like structures—evolutionarily conserved prokaryotic surface organelles. *Curr Opin Microbiol* **14**, 357–363.
- Reindl, S., Ghosh, A., Williams, G. J., Lassak, K., Neiner, T., Henche, A. L., Albers, S. V. & Tainer, J. A. (2013). Insights into FlaI functions in archaeal motor assembly and motility from structures, conformations, and genetics. *Mol Cell* **49**, 1069–1082.
- Senesi, S., Ghelardi, E., Celandroni, F., Salvetti, S., Parisio, E. & Galizzi, A. (2004). Surface-associated flagellum formation and swarming differentiation in *Bacillus subtilis* are controlled by the *ifm* locus. *J Bacteriol* **186**, 1158–1164.
- Siutkin, A. S., Piatibratov, M. G., Beznosov, S. N. & Fedorov, O. V. (2012). [Various mechanisms of flagella helicity formation in Haloarchaea]. *Mikrobiologiya* **81**, 620–629 (in Russian) Medline
- Soppa, J., Baumann, A., Brenneis, M., Dambeck, M., Hering, O. & Lange, C. (2008). Genomics and functional genomics with Haloarchaea. *Arch Microbiol* **190**, 197–215.
- Streif, S., Staudinger, W. F., Marwan, W. & Oesterhelt, D. (2008). Flagellar rotation in the archaeon *Halobacterium salinarum* depends on ATP. *J Mol Biol* **384**, 1–8.
- Szabó, Z., Stahl, A. O., Albers, S. V., Kissinger, J. C., Driessen, A. J. & Pohlschröder, M. (2007). Identification of diverse archaeal proteins with class III signal peptides cleaved by distinct archaeal prepilin peptidases. *J Bacteriol* **189**, 772–778.
- Tarasov, V. Y., Pyatibratov, M. G., Tang, S. L., Dyll-Smith, M. & Fedorov, O. V. (2000). Role of flagellins from A and B loci in flagella formation of *Halobacterium salinarum*. *Mol Microbiol* **35**, 69–78.
- Thomas, N. A. & Jarrell, K. F. (2001). Characterization of flagellum gene families of methanogenic archaea and localization of novel flagellum accessory proteins. *J Bacteriol* **183**, 7154–7164.
- Thomas, N. A., Bardy, S. L. & Jarrell, K. F. (2001). The archaeal flagellum: a different kind of prokaryotic motility structure. *FEMS Microbiol Rev* **25**, 147–174.
- Trachtenberg, S. & Cohen-Krausz, S. (2006). The archaeobacterial flagellar filament: a bacterial propeller with a pilus-like structure. *J Mol Microbiol Biotechnol* **11**, 208–220.
- Tripepi, M., Imam, S. & Pohlschröder, M. (2010). *Haloferax volcanii* flagella are required for motility but are not involved in PibD-dependent surface adhesion. *J Bacteriol* **192**, 3093–3102.
- Tripepi, M., You, J., Temel, S., Önder, O., Brisson, D. & Pohlschröder, M. (2012). N-glycosylation of *Haloferax volcanii* flagellins requires known Agl proteins and is essential for biosynthesis of stable flagella. *J Bacteriol* **194**, 4876–4887.
- Wirth, R. (2012). Response to Jarrell and Albers: seven letters less does not say more. *Trends Microbiol* **20**, 511–512.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Edited by: W. Meijer