**Haloferax volcanii** cells lacking the flagellin FlgA2 are hypermotile

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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; Pohlschroder 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 &

Archael 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 bottom of the flagellar structure in a process that involves FlaI and FlaJ, which are homologous to type IV pilus.

Like type IV pili, which generally contain major pilins that are the primary structural component of the bacterial type IV pili, 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 ΔflgA2 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 ΔflgA2 (Hvo_1212) deletion mutant and flgA2Stop 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**

<table>
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<th>Plasmid/strain</th>
<th>Relevant properties</th>
<th>Reference</th>
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<tr>
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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 (FlgA1–2His), 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 FlgA1–2, respectively) are listed in Table S1. pMT10 and pMT21 were isolated from E. coli DH5α 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 QuickChange 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 DH5α. 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). 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 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 semidy 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 ΔflgA2, 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 (Hertzog & Wirth, 2012), or the macerate was subjected to a 15 s low-speed centrifugation to pellet most of the soft agar and the supernatant was analysed as previously described (Hertzog & Wirth, 2012) and analysed using ImageJ. The mean speed of five different cells was recorded for each experiment for both WT and ΔflgA2, 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 21 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 16000 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 50000 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.

**RESULTS**

**Transcription of flgA2 in a ΔflgA1 strain**

We have previously shown that an Hfx. volcanii ΔflgA1 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 ΔflgA1 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 ΔflgA2 and ΔflgA2Stop 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 ΔflgA2 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 ΔflgA2 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. 1). Time-compressed videos (2 frames s⁻¹) of ΔflgA2 and ΔflgA2Stop strains were recorded as in FlgA1Δ WT H53 strain. However, ΔflgA2 and ΔflgA2Stop strains can also be grown on plates of 0.5 % agar MGM on which they exhibit a hypermotile phenotype (Fig. 1).
The phenotype is not caused by an increased growth rate for wild-type and CA medium, suggesting that this hypermotility phenotype is not a result of a higher growth rate. To determine whether the increase in motility is due to a transcriptional or post-transcriptional regulation of flgA2, we constructed a strain in which the chromosomal copy of flgA2 was replaced with a stop codon (TGA) (Fig. 3b).

With the previously described extension of our strain collection, we now have a ΔflgA1ΔflgA2 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 ΔflgA1 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 ΔflgA2 strain (see above).

Additionally, we have created three pTA963-based expression plasmids: one construct co-expresses an unmodified FlgA1 and a His-tagged FlgA2 [flgA1–2]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 ΔpilB3C3 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. Pohlschro¨der, 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 ΔflgA1 and ΔflgA1ΔflgA2 strains can be complemented by expressing flgA1His and flgA1–2His 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 ΔflgA1 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 ΔflgA1 strain and does not rescue the defect of the ΔflgA1ΔflgA2 strain, while co-expression of FlgA1–2His 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 FlgA1–2His 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 ΔflgA1 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 ΔflgA1 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 ΔflgA2 strain is intriguing. We analysed two configurations in which only the FlgA1 flagellin is expressed; when expressing FlgA1His in a ΔflgA1–2 strain, no motility is observed. However, a ΔflgA2 strain expressing only FlgA1 is hypermotile. This, as well as the fact that expression of FlgA2 in trans does not
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. Pohlschroeder, 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. Pohlschroeder, 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 ΔpilB3C3ΔflgA1–2 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 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.

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However, strikingly, while the ΔpilB3C3 strain generally had one or two flagella associated with single cells, the ΔpilB3C3ΔflgA2 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 ± 2.6 μm compared with 1.8 ± 1.1 μm in the ΔpilB3C3 strain. Consistent with these data, we observed significantly more flagella structures in the culture supernatant of ΔpilB3C3ΔflgA2 cells compared to that of the ΔpilB3C3 strain expressing flgA2 (Fig. 5b).

ΔflgA2 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 ΔflgA2 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 ΔflgA2 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. 4. Flagellins expressed in Hfx. volcanii WT, and ΔflgA1, ΔflgA2 and ΔflgA[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.](http://mic.sgmjournals.org/)

![Fig. 5. Differential expression of flagella in ΔflgA1 and ΔflgA2. (a) Electron micrographs of ΔpilB3C3, ΔpilB3C3ΔflgA[1–2], ΔpilB3C3ΔflgA1 and ΔpilB3C3ΔflgA2 cells fixed in 2 % glutaraldehyde and 1 % paraformaldehyde followed by negative staining with 1 % uranyl formate. Bars, 200 nm. (b) Electron micrographs of ΔpilB3C3, ΔpilB3C3ΔflgA[1–2], ΔpilB3C3ΔflgA1 and ΔpilB3C3ΔflgA2 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.](http://mic.sgmjournals.org/)
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 ΔflgA1–2 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 flgA1–2 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 ΔflgA1–2 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 flgA1–2 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.

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