Molecular Microbiology (2014) 93(3), 494–504 ■
doi:10.1111/mmi.12673
First published online 9 July 2014

A conserved type IV pilin signal peptide H-domain is critical for the post-translational regulation of flagella-dependent motility

Rianne N. Esquivel and Mechthild Pohlschroder*
Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA.

Summary

In many bacteria and archaea, type IV pili facilitate surface adhesion, the initial step in biofilm formation. *Haloferax volcanii* has a specific set of adhesion pilins (PilA1-A6) that, although diverse, contain an absolutely conserved signal peptide hydrophobic (H) domain. Data presented here demonstrate that these pilins (PilA1-A6) also play an important role in regulating flagella-dependent motility, which allows cells to rapidly transition between planktonic and sessile states. Cells lacking adhesion pilins exhibit a severe motility defect, however, expression of any one of the adhesion pilins in trans can rescue the motility and adhesion. Conversely, while deleting *pilB3-C3*, genes required for PilA pilus biosynthesis, results in cells lacking pili and having an adhesion defect, it does not affect motility, indicating that motility regulation requires the presence of pilins, but not assembled pili. Mutagenesis studies revealed that the pilin-dependent motility regulatory mechanism does not require the diverse C-terminal region of the PilA pilins but specifically involves the conserved H-domain. This novel post-translational regulatory mechanism, which employs components that promote biofilm formation to inhibit motility, can provide a rapid response to changing environmental conditions. A model for this regulatory mechanism, which may also be present in other prokaryotes, is discussed.

Introduction

Biofilms are complex microbial communities, bound by a matrix of extracellular polymeric substance (EPS) that allow cells to tolerate stress conditions such as high UV exposure (Hansen *et al.*, 2007; Monds and O'Toole, 2009; Haussler and Fuqua, 2013; Orell *et al.*, 2013a). Once environmental conditions become unfavourable, cells must rapidly transition from a planktonic to a sessile state (McDougald *et al.*, 2011). For instance, flagella are required for the swimming motility of planktonic cells and enhance initial type IV pilus-dependent attachment of many bacteria and archaea to surfaces (O'Toole and Kolter, 1998; Ghosh and Albers, 2011). However, flagella are not required, and indeed hinder, subsequent stages of biofilm development whereas adhesive pili are crucial to the formation of cell aggregates in many prokaryotic biofilms (Fröls *et al.*, 2008; Karatan and Watnick, 2009; Pohlschroder *et al.*, 2011; Esquivel *et al.*, 2013).

Several strategies to inactivate already existing flagella and to inhibit the biosynthesis of new flagella have evolved (Guttenplan and Kearns, 2013). For example, in *Bacillus subtilis* the glycosyltransferase, EpsE, which is required for matrix biosynthesis, binds to the flagella rotor and disengages motor force-generating elements, providing a rapid mechanism for inhibiting flagella rotation (Blair *et al.*, 2008). High levels of c-di-GMP, produced by many bacteria during biofilm formation, also inhibit transcription of genes that encode flagella biosynthesis components, and upregulate the expression of genes involved in adhesive pili formation. Conversely, once a biofilm begins to disperse, a condition where pili expression becomes disadvantageous, c-di-GMP levels decrease, resulting in repression of *pil* genes and increased *fla* gene expression (Kuchma *et al.*, 2007; Boyd and O'Toole, 2012; Guttenplan and Kearns, 2013).

Interestingly, unlike the analogous surface filaments of bacteria, which are produced by distinct biosynthesis machineries, archaeal pili and flagella (the latter also known as archaella; Jarrell and Albers, 2012) are assembled by machineries that use homologous, or even share, core components (Ghosh and Albers, 2011; Pohlschroder *et al.*, 2011). These components are also homologous to proteins involved in bacterial pilus biosynthesis. As such, flagellins and pilins in many archaeal species are processed by the same PilD homologue, PibD (FlaK) (Albers *et al.*, 2003; Bardy and Jarrell, 2003; Giltner *et al.*, 2012). Unlike signal peptidase I and II which have recognition sites that follow the signal peptide hydrophobic (H) domain,

Accepted 13 June, 2014. *For correspondence. E-mail pohlschr@sas.upenn.edu; Tel. (+1) 215 573 2283; Fax (+1) 215 898 8780.

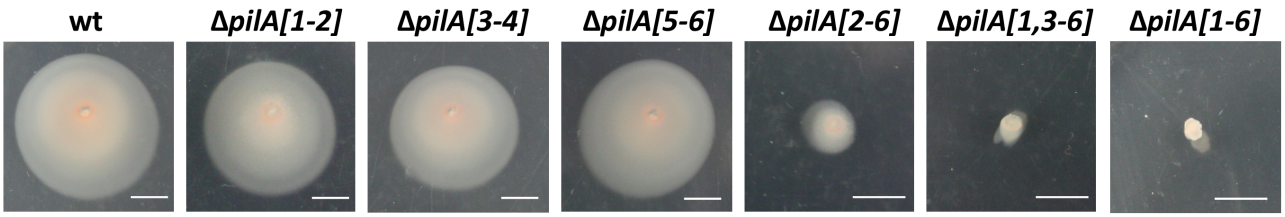

Fig. 1. Deleting at least five pilin genes impairs flagella-dependent motility. Motility assays of *H. volcanii* wild-type (wt) and *pilA* deletion strains after three days of incubation in MGM. Bars, 5 mm.

the PibD recognition sites in type IV pilins precede the H-domain and the processed pilins and flagellins are thought to be retained in the membrane prior to assembly. In the mature protein, this H-domain forms an alpha helix that is predicted to serve as the core of the flagella or pili (Craig *et al.*, 2006; Pohlschroder *et al.*, 2011; Giltner *et al.*, 2012). Archaeal pili and flagella assembly also requires the presence of homologues of the pilus biosynthesis ATPase PilB and of the membrane protein PilC (Takhar *et al.*, 2013). However, distinct PilB and PilC paralogues are required for archaeal type IV pilus and flagella biosynthesis (Albers and Pohlschroder, 2009; Ghosh and Albers, 2011; Lassak *et al.*, 2012). Consistent with having unique biosynthesis machineries, it has recently been shown that the transcriptional regulation of archaeal flagella and pili expression within biofilms is, as in bacteria, inversely related (Reimann *et al.*, 2012; Lassak *et al.*, 2013; Orell *et al.*, 2013b).

While recent advancements have been made in understanding the relationship between the transcriptional regulation of flagella and pili, little is known about the post-transcriptional regulation of these archaeal filaments. In the euryarchaeon *Haloferax volcanii*, which expresses two flagellin genes, *flgA1* and *flgA2*, the deletion of the gene encoding the flagellar subunit, *flgA2*, results in hypermotility (Tripepi *et al.*, 2013). The effect that deletion of a flagellar subunit leads to hypermotility is unprecedented, but the regulatory mechanism underlying this effect has not yet been determined.

H. volcanii is also the first motile organism to be reported where the initial adhesion to a surface does not require flagella (Tripepi *et al.*, 2010) and where a subset of six identified adhesion pilins (PilA1-A6) are required for microcolony formation, while others appear to inhibit this early step in biofilm formation (Esquivel *et al.*, 2013). Finally, each of these pilins, even though they are rather diverse, has a completely conserved H-domain that is required for the assembly of a pilus (Szabó *et al.*, 2007; Esquivel *et al.*, 2013).

In this study, we have shown that the PilA adhesion pilins play essential roles in the post-translational regulation of flagella-dependent motility and that the absolutely conserved H-domain specific to these adhesion pilins is essential to this regulatory function. Although this additional pilin function, the third pilin function identified that is involved in processes required for biofilm formation, has not been reported previously, it may not be unique to halophiles.

Results and discussion

ΔpilA[1-6] has a severe motility defect

Considering the hypermotility phenotype observed in *Sulfolobus acidocaldarius* lacking pilus biosynthesis genes *aapE*, *aapF* or the major pilin gene, *aapB* (Henche *et al.*, 2012) we stab-inoculated deletion mutants, lacking either single or multiple pilins, on motility plates and determined their motility phenotypes. Our results show that the deletion of a single *H. volcanii pilA* gene or two *pilA* genes does not significantly affect motility compared to that of the H53 parent strain (from hereon referred to as the wild-type) (Fig. 1). Unexpectedly, while these mutants (Δ*pilA1*, Δ*pilA2*, Δ*pilA*[*1-2*], Δ*pilA*[*3-4*], or Δ*pilA*[*5-6*]) and the wild-type strain form a visible halo after two days, a deletion strain expressing only chromosomally encoded PilA1 exhibited decreased motility, only forming a visible halo after three days (Fig. 1 and data not shown). This mutant, Δ*pilA*[*2-6*], also displays a severe adhesion defect (Esquivel *et al.*, 2013). Moreover, the non-adhering mutant strain expressing only chromosomal PilA2 and the mutant strain lacking all six *pilA* genes do not show any significant motility even after five days of incubation (Fig. 1 and Fig. S1). This is consistent with both of these mutants, Δ*pilA*[*1,3-6*] and Δ*pilA*[*1-6*], being unable to adhere (Esquivel *et al.*, 2013). These results strongly suggest that *H. volcanii* swimming motility is regulated in a pilin-dependent manner through a novel regulatory mechanism not previously identified.

The majority of ΔpilA[1-6] cells are non-flagellated

Motility, adhesion and filament assembly phenotypes of *H. volcanii* wild-type and mutant strains discussed in this manuscript are summarized in Table 1. The inhibited and delayed motility observed for the Δ*pilA*[*1-6*] strain might be due to the fact that: (1) only a few cells have functional

Table 1. Motility, filament assembly and adhesion phenotypes exhibited by *H. volcanii* wt and mutant strains.

Strain	Plasmid	Motility[a]		Filaments	Adhesion	Reference
			EM	α-His(cell/CsCl)		
wt	–	+++	+++	NA	+++	Tripepi *et al.* (2012)
Δ*pilA[1-6]*	–	–	–	NA	–	This study/Esquivel *et al.* (2013)
Δ*pilA[1-6]*	PilA1His	+++	++[b]	++/–	++	This study/Esquivel *et al.* (2013)
Δ*pilA[1-6]*	PilA1HybHis	–	+	–/–	–	This study/Esquivel *et al.* (2013)
Δ*pilA[1-6]*	FlgA1HybHis	++	++	ND	–	This study
Δ*pilA[1-6]*	FlgA1His	–	ND	ND	–	This study
Δ*flgA[1-2]*	–	–	+++	NA	+++	Tripepi *et al.* (2012)
Δ*flgA[1-2]*	FlgA[1-2]His	+++	+++	+++/ND	+++	Tripepi *et al.* (2012)
Δ*pilA[1-6]*Δ*flgA[1-2]*	–	–	–	NA	–	This study
Δ*pilA[1-6]*Δ*flgA[1-2]*	FlgA[1-2]His	–	+	+/–	–	This study
Δ*pilB3-C3*	–	+++	+++	NA	+	This study/Tripepi *et al.* (2013)
Δ*pilB3-C3*	PilB3-C3His	+++	+++	NA	++++	This study/Tripepi *et al.* (2013)
Δ*pilB3-C3*Δ*flgA[1-2]*	–	–	–	NA	+	This study
Δ*flgA1*	–	–	++	NA	+++	Tripepi *et al.* (2013)
Δ*flgA1*	FlgA1His	+++	+++	++/++	+++	Tripepi *et al.* (2013)
Δ*flgA1*	FlgA1HybHis	–	++	–/ND	+++	This study
Δ*pilB3-C3*Δ*flgA1*[c]	–	++	+	NA	+	This study
Δ*pilB3-C3*Δ*flgA1*	FlgA1His	++	ND	++/ND	+	This study
Δ*pilB3-C3*Δ*flgA1*	FlgA1HybHis	++	ND	++/–	+	This study
Δ*flgA2*	–	++++	++++	NA	+++	Tripepi *et al.* (2013)
Δ*pilA[1-6]*Δ*flgA2*	–	–	ND	NA	–	This study

a. Motility after 3 days of incubation.
b. Pili were only detected associated with the cells.
c. Only motile on semi-defined media.

flagella; or (2) the flagella are not fully functional in this mutant background. To distinguish between these possibilities, we compared *H. volcanii* Δ*pilA*[*1-6*] with the wild-type strain using transmission electron microscopy (TEM). While about 40% of the wild-type cells have filaments associated with them, filamentous surface structures were observed on only 2 of approximately 100 Δ*pilA*[*1-6*] cells analysed (Fig. 2A).

We have previously demonstrated that flagella and pili are released into the culture supernatants upon centrifugation (Tripepi *et al.*, 2012). To determine whether the Δ*pilA*[*1-6*] cells produce flagella that are unstable and shed, even under the mild conditions used for TEM preparations, we analysed culture supernatant fractions of the wild-type, Δ*flgA*[*1-2*] and Δ*pilA*[*1-6*] strains using cesium chloride (CsCl) gradient centrifugation. While TEM cannot

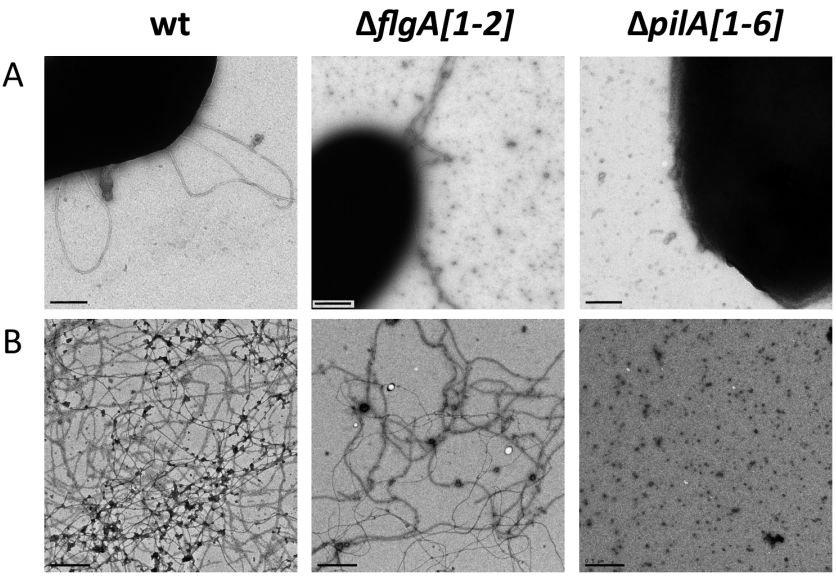

Fig. 2. Only a small subset of cells lacking PilA1-A6 contain flagella.
A. TEM of whole cells of wt and deletion strains. Bars, 200 nm. Images represent approximately 40% of wt and Δ*flgA*[*1-2*] cells and 98% of Δ*pilA*[*1–6*] cells analysed.
B. TEM of surface filaments purified by CsCl gradient centrifugation. Bars, 0.5 μm.

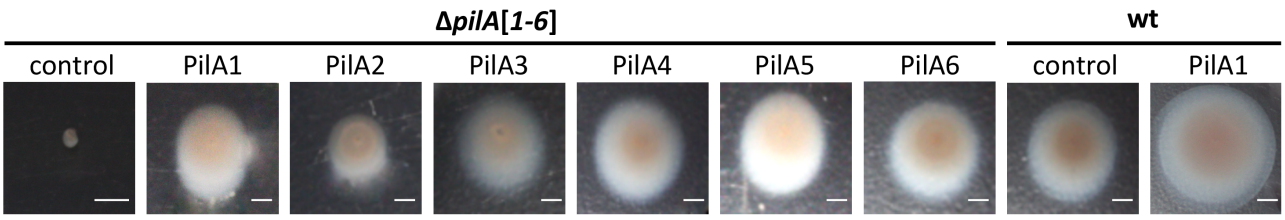

Fig. 3. One PilA pilin is sufficient to complement the *H. volcanii* Δ*pilA[1–6]* motility defect. Motility assays of *H. volcanii* Δ*pilA[1-6]* strain transformed with pTA963 (control) or pTA963 encoding a His-tagged version of one of the six pilin genes under the regulation of a *trp*-inducible promoter or wt transformed with pTA963 or pTA963 encoding *pilA1His*. Bars, 5 mm.

distinguish between flagella and pili, examination of the CsCl gradient purified filaments revealed that the Δ*flgA*[*1-2*] culture supernatant contains a significantly reduced number of filaments compared to the wild-type, indicating that an ample portion of the filaments produced by the wild-type are flagella (Fig. 2B). Conversely, in the Δ*pilA*[*1-6*] fractions, no filaments were detected. These observations indicate a significantly decreased synthesis or stability of flagella when pilin genes are absent (Fig. 2B).

Each of the six PilA pilins can complement the motility defect of the ΔpilA[1-6] strain

The motility phenotypes of the Δ*pilA* strains suggest that effective flagella-dependent motility requires the presence of at least some PilA pilins (Fig. 1). Consistent with the delayed motility phenotype observed for a Δ*pilA*[*1-6*] strain being due to the absence of all PilA adhesion pilins, this defect can be rescued by the expression of any one of the six PilA pilins in trans (Fig. 3). While the Δ*pilA*[*1-6*] deletion strain expressing PilA2 is somewhat less motile than the wild-type, the expression of PilA1, 3, 4, 5 or 6 in trans fully rescues the Δ*pilA*[*1-6*] motility defect. Similarly, PilA2 only partially rescues the adhesion defect of the Δ*pilA*[*1-6*] strain (Esquivel *et al*., 2013). Additionally, expression of each of the pilins individually in trans in a wild-type strain results in a hypermotility phenotype, supporting our hypothesis that a novel mechanism, involving PilA pilins, regulates *H. volcanii* flagella-dependent motility (Fig. 3 and Fig. S2). Since the expression of a single adhesion pilin gene can successfully complement this motility defect, this regulatory mechanism might operate in other archaea or bacteria but may yet have gone unnoticed.

PilA dependent regulation of motility is post-translational

We previously determined that FlgA[1-2]His can complement the motility defect of a Δ*flgA*[*1-2*] strain when expressed in trans from a *trp* inducible promoter (Large *et al*., 2007; Tripepi *et al*., 2010). However, expressing this construct in the Δ*pilA*[*1-6*]Δ*flgA*[*1-2*] strain does not complement the motility defect, indicating that the lack of pili may have a post-translational effect (Fig. 4A). While raising antibodies against *H. volcanii* flagellins and pilins has proven difficult, FlgA2His can be readily detected in cell extracts by using an anti-His antibody in Western blot analyses (Fig. 4B). However, only a faint band was identified in Western blots of protein from the supernatant fractions. To confirm these results we used CsCl gradient centrifugation to isolate flagella in a large-scale preparation from 1 l of culture supernatant of the Δ*pilA*[*1-6*]Δ*flgA*[*1-2*] strain expressing FlgA[1-2]His from the *trp* inducible promoter. FlgA2His was not detected in this preparation (Fig. 4B). In fact, consistent with flagella being unstable in these cells, none of the approximately 100 cells analysed by TEM had surface filaments (Fig. 4C) and only two small,

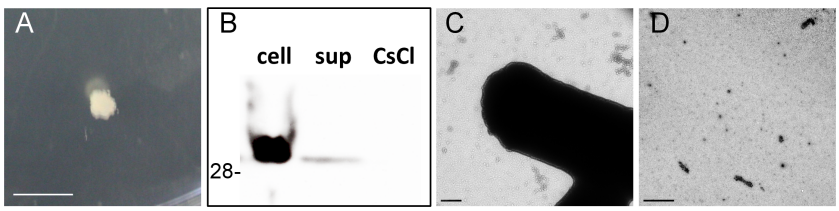

Fig. 4. Lack of pilins results in post-translational regulation of flagella-dependent motility. Δ*pilA[1-6]*Δ*flgA[1-2]* strain expressing *flgA[1-2]His* under the regulation of a *trp*-inducible promoter were analysed by (A) motility assay, (B) anti-His Western blot, and TEM of (C) whole cells or (D) filaments purified using CsCl gradient centrifugation. The Westerns were performed on protein extracts from cell lysates, TCA-precipitated proteins from culture supernatants (sup), or CsCl gradient purifications of surface filaments. Comparable culture volumes were used in cell and sup protein preparations. Molecular mass standard indicated on the left (in kDa). Bars, 5 mm (motility assay), 200 nm (cell) or 0.5 μm (CsCl).

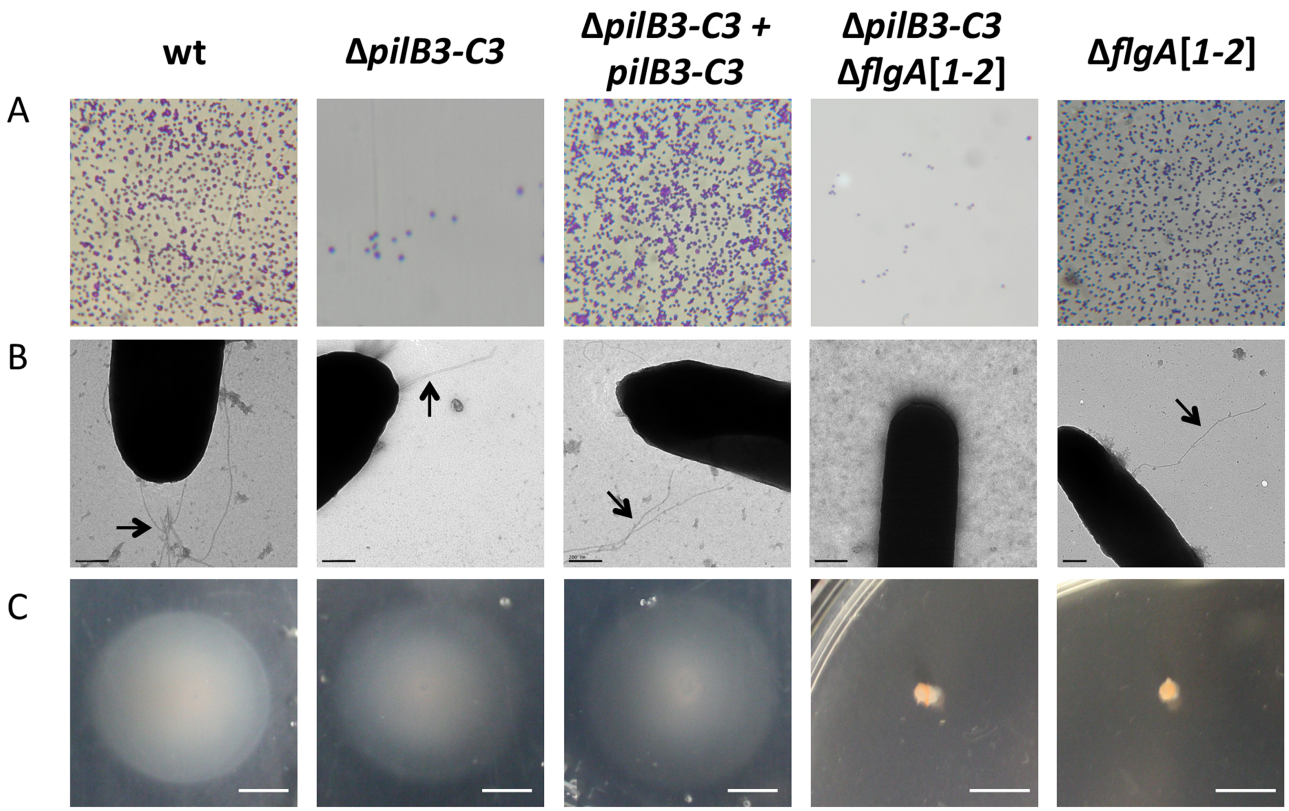

Fig. 5. Pilus assembly is not required for motility. (A) Surface adhesion assays (× 35 magnification), (B) TEM of whole cells and (C) motility assays of wt, Δ*pilB3-C3*, Δ*pilB3-C3*Δ*flgA[1-2]* and Δ*flgA[1-2]* strains transformed with pTA963 and Δ*pilB3-C3* transformed with pTA963 encoding *pilB3-C3His*. Surface filaments are observed on wt, Δ*pilB3-C3*, Δ*pilB3-C3* expressing PilB3-C3 and Δ*flgA[1-2]* strains (arrows). Bars, 5 mm (motility assay) or 200 nm (cell).

unattached filaments were observed in the surrounding supernatant. Moreover, no filaments were detected in CsCl gradient fractions (Fig. 4D). Taken together, these results strongly suggest that this pilin-dependent regulation of flagella biosynthesis is, at least in part, post-translational.

H. volcanii *PilB3 and PilC3 are required for PilA pilus biosynthesis*

We previously showed that the expression of any one of the six PilA pilins in a Δ*pilA*[*1-6*] strain results in the synthesis of pili (Esquivel *et al.*, 2013). To address whether flagella-dependent motility requires the presence of pili or whether pilins alone are sufficient, it was critical to identify pilus biosynthesis components of these PilA adhesion pili. Since the *H. volcanii* genome has five *pilB* and *pilC* containing operons in addition to its paralogues *flaI* and *flaJ*, respectively, in the flagella biosynthesis operon (Hartman *et al.*, 2010), our first objective was to identify the PilB/C paralogues required for the PilA adhesion pilus biosynthesis.

Consistent with the observed colocalization of *pilB* and *pilC* with pilin genes in many characterized *pil* operons, all *H. volcanii pilB* and *pilC* containing operons, except for the *pilB3-C3* containing operon, colocalize with at least one gene that encodes a predicted pilin-like protein (Fig. S3), a protein containing a pilin cleavage site as predicted by the program FlaFind (Szabó *et al.*, 2007). Since none of the conserved *pilA1-A6* pilin genes are associated with a *pilB* or *pilC* gene, we hypothesized that PilB3-C3 might be involved in PilA pilus biosynthesis.

We generated an *H. volcanii* deletion strain lacking *pilB3* and *pilC3* (Δ*pilB3-C3*) using homologous recombination (Allers and Ngo, 2003). The deletion was confirmed by PCR using primers homologous to sequences lying just outside these genes (Fig. S4A, Table S2) as was done previously for all other knockout strains (Esquivel *et al.*, 2013). The Δ*pilB3-C3* strain was tested for adhesion to plastic coverslips and it was found that it has a severe adhesion defect, indicating that the pilus-biosynthesis components PilB3-C3 are required for PilA adhesion pili biosynthesis (Fig. 5A). Consistent with a defect in pilus biosynthesis in this strain, when examined by TEM, only pili, not flagella, can be detected on a Δ*flgA[1-2]* strain, as previously determined (Tripepi *et al.*, 2012), while introducing a *pilB3-C3* deletion into this

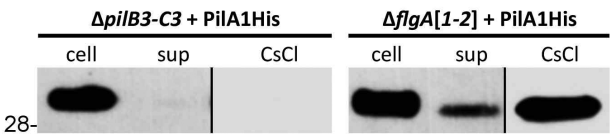

Fig. 6. PilA pilins are not incorporated into flagella. Western blot analysis using anti-His antibodies was performed on protein extracts from cell lysates (cell), TCA-precipitated proteins from the supernatant (sup), or CsCl gradient purifications of the surface filaments from the Δ*pilB3-C3* or Δ*flgA[1-2]* strains expressing PilA1His. Molecular mass standard indicated on the left (in kDa).

strain results in bald cells (Fig. 5B). Expression of PilB3-C3 in trans restored adhesion and pilus biosynthesis (Fig. 5A and B). These results also suggest that the limited level of adhesion observed in the Δ*pilB3-C3* strain is not due to residual assembled pili. Perhaps membrane-associated pilins are capable of mediating some adhesion.

Pili are not required for H. volcanii *motility*

Since PilB3-C3 are required for PilA pilus biosynthesis, we could use a Δ*pilB3-C3* strain to determine whether an assembled pilus is required to affect flagella-dependent motility or whether pilins alone are sufficient. When stab-inoculated into motility plates, the Δ*pilB3-C3* mutant displays motility similar to that of the wild-type strain, suggesting that assembled pili are not required for motility (Fig. 5C). Consistent with these results, while a Δ*pilA*[*1-6*] strain is bald, lacking surface-associated flagella as well as pili (Fig. 2A), a Δ*pilB3-C3* strain does have surface filaments, as determined by TEM (Fig. 5B). As noted above TEM does not distinguish between flagella and pili. However, considering that a Δ*pilB3-C3*Δ*flgA*[*1-2*] strain is bald (Fig. 5B), these data indicate that the filaments observed on this strain are flagella. To confirm that filaments observed in a Δ*pilB3-C3* background are flagella and that this strain is unable to assemble pili, we overexpressed PilA1His in the Δ*pilB3-C3* and the Δ*flgA[1-2]* strains. Using anti-His antibodies, Western blot analyses readily detected PilA1His in protein extracts from cell preparations of both strains, while this tagged pilin subunit can only be detected in supernatant fractions of cells that have functional PilB3-C3 (Fig. 6). Immunogold labelling of pili including His-tagged pilins was attempted but not successful, possibly due to the high salt concentration used or the His-tag being buried within the structure. However, lack of PilA1His in the supernatant of a Δ*pilB3-C3* strain was confirmed by Western blot analysis of a CsCl gradient purified protein preparation derived from 1 l of culture (Fig. 6). The absence of any His signal in the concentrated pili/flagella preparation of the Δ*pilB3-C3* supernatant fraction not only shows that pili are not formed but also strongly suggests that PilA1His is not incorporated into flagella and that the regulation of motility is mediated by membrane associated pilins.

The conserved H-domain, but not the pilin, is critical for motility regulation

Having established that pilin subunits, but not the pili, are required for regulation of motility, we set out to determine which parts of the pilin are important in regulating motility. We have shown that expression of a hybrid pilin, PilA1Hyb, containing the FlgA1 signal peptide hydrophobic stretch, instead of the conserved PilA H-domain, does not rescue the adhesion defect of the Δ*pilA*[*1-6*] strain nor does it result in the biosynthesis of pili in this deletion strain (Fig. 7A; Esquivel *et al.*, 2013). To assess the importance of the conserved H-domain for motility regulation, we stab-inoculated the Δ*pilA*[*1-6*] strain expressing PilA1Hyb in

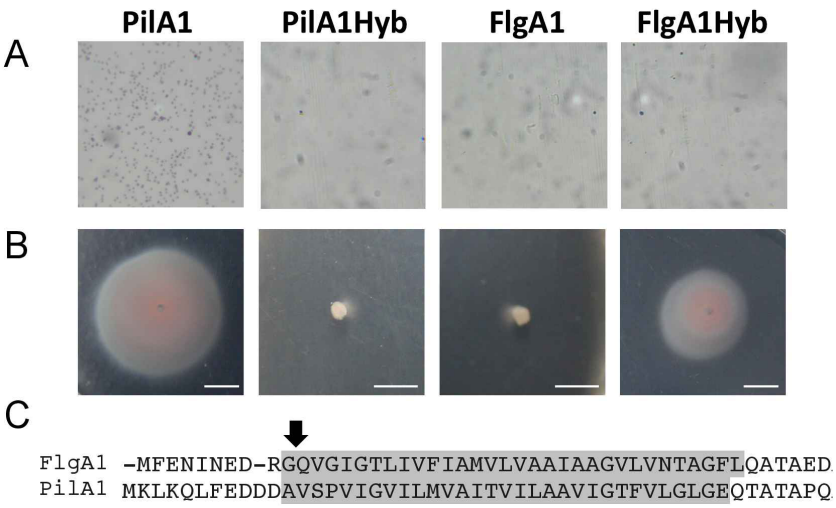

Fig. 7. The PilA1-A6 conserved H-domain is critical for pilin-dependent motility regulation.
A and B. (A) Adhesion assays (×35 magnification) and (B) motility assays of *H. volcanii* Δ*pilA*[*1-6*] strain transformed with pTA963 encoding *pilA1His*, *pilA1HybHis*, *flgA1His* or *flgA1HybHis*. A spot on the microscope lens is seen in FlgA1 and FlgA1Hyb.
C. N-terminal amino acid sequence of *H. volcanii* FlgA1 and PilA1. PilA1 H-domain used to construct the FlgA1Hybrid and FlgA1 H-domain replaced in that flagellin hybrid, are highlighted in gray. Arrow indicates predicted PibD processing site. Bars, 5 mm.

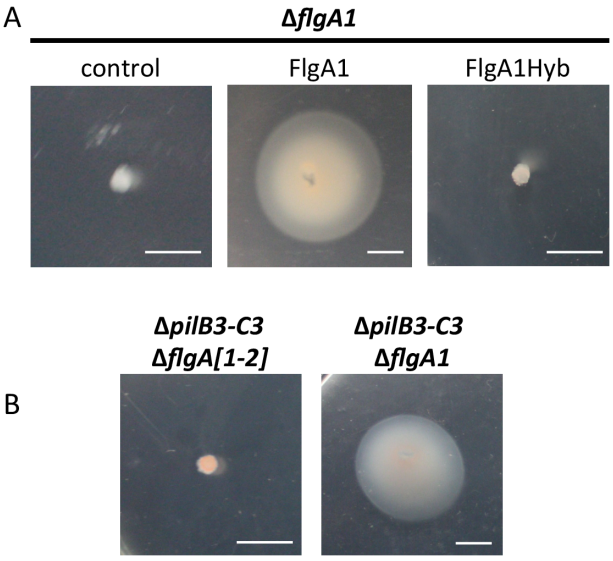

Fig. 8. FlgA1Hyb cannot make functional flagella.
A. Motility assays of Δ*flgA1* strain transformed with pTA963 (control) or pTA963 encoding *flgA1His* or *flgA1HybHis*.
B. Motility assays of Δ*pilB3-C3*Δ*flgA[1-2]* and Δ*pilB3-C3*Δ*flgA1* transformed with pTA963, demonstrating that FlgA2 can support motility in an FlgA1-independent manner, in the absence of *pilB3-C3*. Bars, 5 mm.

trans into motility plates. Unlike PilA1 expressed in trans this hybrid construct cannot complement the Δ*pilA*[*1-6*] motility defect, suggesting that multiple pilin functions critically depend on the H-domain (Fig. 7B).

To determine whether the hydrophobic stretch can only regulate motility in the context of a mature pilin, we created a hybrid flagellin containing the conserved pilin H-domain (Fig. 7C). When expressed in a Δ*pilA*[*1-6*] background, this hybrid protein, FlgA1Hyb, as expected, does not rescue the adhesion defect (Fig. 7A). However, it does rescue motility in the Δ*pilA*[*1-6*] strain, albeit not to wild-type levels, indicating the conserved H-domain can regulate swimming motility independent of the C-terminal pilin sequence (Fig. 7B).

To confirm that the His-tagged FlgA1Hyb construct could not make functional flagella, we expressed this hybrid flagellin in the Δ*flgA1* strain, which is non-motile. We have previously shown that this deletion strain can be complemented by expressing FlgA1His in trans (Tripepi *et al*., 2010). Unlike FlgA1His, expression of FlgA1HybHis does not complement the motility defect in the Δ*flgA1* strain (Fig. 8A), probably because the PilA H-domain is not compatible with the flagella assembly machinery.

Surprisingly, a Δ*flgA1* strain became motile upon the deletion of *pilB3-C3* when grown on semi-defined media rather than complex media (Fig. 8B and Tripepi *et al*., 2013). Possibly, similar to the overexpression of pilins, deletion of pilus-biosynthesis genes results in an increased number of pilins in the membrane, hence promoting motility, and allowing FlgA2, in the absence of FlgA1 to confer motility. Multiple mechanisms seem to be involved in regulating *H. volcanii* motility. This could explain why an increase in motility is not observed in a Δ*pilB3-C3* strain but is observed upon the deletion of *pilB3-C3* and *flgA1* (Fig. 8B) or overexpression of pilins in the wild-type strain (Fig. 3 and Fig. S2). While a Δ*flgA2* mutant is hypermotile, suggesting a regulatory role for this flagellin (Tripepi *et al*., 2013), these results demonstrate that FlgA2 is sufficient to make functional flagella, which has not been observed before.

FlgA2 and pilin motility regulation are independent

Considering the Δ*flgA2* hypermotility phenotype and the pilin-like signal peptide structure of archaeal flagellins, we had previously hypothesized that the PilA H-domain might interact with the FlgA2 H-domain in the cell membrane, sequestering inhibitory concentrations of FlgA2. While this FlgA1-independent motility indicates that the FlgA2 and pilin-dependent motility regulation are independent, these results do not exclude the possibility that pilin-FlgA2 interactions are occurring in the presence of FlgA1. If the Δ*pilA*[*1-6*] strain had a severe motility defect because FlgA2 was not sequestered, the Δ*pilA*[*1-6*] in which *flgA2* was also deleted should be motile. However, our results show that the deletion of the six pilins in the absence of FlgA2 (Fig. S4B), still results in a severe motility defect, supporting two novel, independent regulatory mechanisms (Fig. 9).

Conclusions

Most prokaryotic cells can reversibly exist as either motile planktonic cells or as sessile cells in biofilms. These cells require flagella to swim in liquid media, and many use type

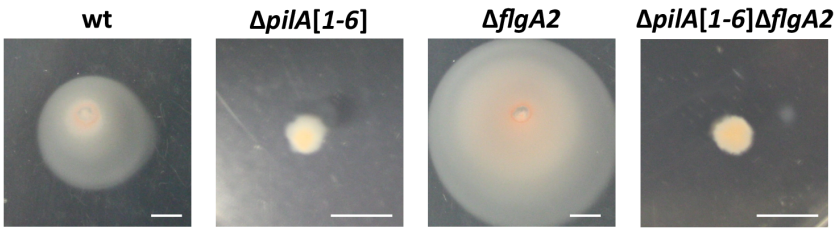

Fig. 9. PilA pilin motility regulation is FlgA2-independent. Motility assays of wt and deletion strains in MGM. Bars, 5 mm.

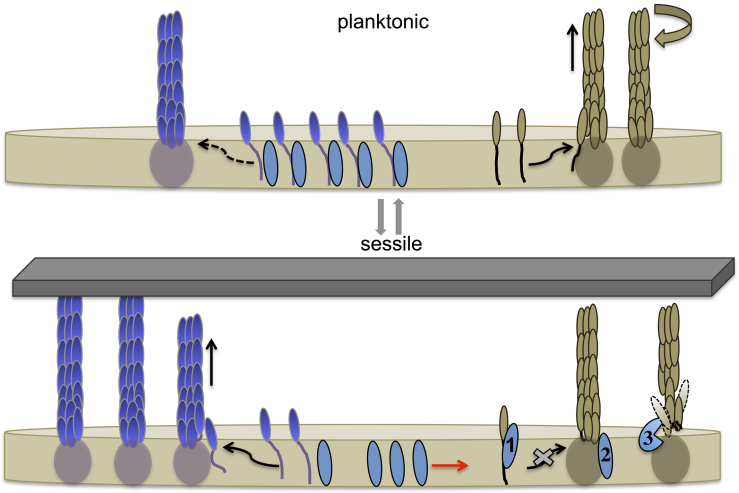

Fig. 10. Model for pilin-mediated inhibition of swimming motility. During planktonic growth *H. volcanii* cells synthesize flagellins that are readily incorporated into flagella, supporting swimming motility. The cells also express pilins, which are incorporated into pili at a slow rate. The H-domain of membrane-associated pilins interacts with, and hence sequesters, a protein that directly or indirectly inhibits flagella motility. Upon adhesion, pilus-assembly kinetics shifts and the affinity of pilins for pili increases, depleting the membrane of pilins and releasing the inhibitor proteins. These inhibitors interfere with flagella biosynthesis and/or stability. Taken together, this allows cells to rapidly respond to environmental conditions that favour biofilm formation rather than motility. Three possible mechanisms through which an inhibitor might obstruct swimming motility are: (1) direct interaction with flagellins, thus preventing the incorporation of subunits into the flagellum; (2) inactivation of a flagella-biosynthesis component(s) or (3) degradation/destabilization of the flagella.

IV pili to mediate attachment to surfaces. Therefore, it is not surprising that cells have evolved transcriptional and post-transcriptional mechanisms to tightly regulate the biosynthesis and function of pili and flagella during the transition between a motile and sessile state. We have demonstrated here, for the first time, that type IV pilins themselves can be involved in the post-translational regulation of flagella-dependent motility. Although other modes of regulating flagella-dependent motility likely play a role in regulating *H. volcanii* motility, the novel post-translational pilin-dependent regulatory mechanism reported in this study is unique in allowing a rapid transition of cells from a sessile to a planktonic state when local environmental conditions change. Our data show that the highly conserved pilin H-domain, which is required for membrane targeting and pilus biosynthesis, is also critical for this motility regulation. It may be that this functional role has resulted in the 100% conservation of its amino acid sequence across the H-domain of all six, otherwise diverse, adhesion pilins. While a construct only expressing the H-domain was unstable, we were able to complement motility using a FlgA1Hyb protein lacking the C-terminal pilin domain. Localization studies of this construct also suggest that the H-domain does not promote motility by incorporating into the flagella structure. Perhaps the adhesion pilins sequester another non-flagellin protein that directly or indirectly inhibits motility. We have also observed that *H. volcanii* synthesizes flagella as well as pili during planktonic growth and that subunits of these surface filaments can be detected in the membrane of planktonic cells (Tripepi *et al.*, 2013). Given these data we propose the model described in Fig. 10.

We were able to identify this regulatory mechanism because the surface adhesion of *H. volcanii* is not affected unless at least five of the six genes encoding adhesion pilins are deleted (Esquivel *et al.*, 2013), which results in a strain having a cell membrane that is depleted of adhesion pilins. To the best of our knowledge, in studies of pili in other prokaryotic systems no attempts have been made to obtain strains depleted of all major and minor adhesion pilins involved in the biosynthesis of pili that are required for surface adhesion. Hence, our results, which suggest a novel system for the regulation of expression of surface filaments during biofilm formation, might also be identified in other archaea and bacteria when the corresponding strains, lacking all pilins involved in adhesion, are generated. A closer examination of domains conserved between pilins might ultimately lead to the determination that this regulatory mechanism is used across a wide variety of prokaryotic species.

Future studies will focus on the identification of the predicted pilin-interacting motility inhibitor using co-immunoprecipitation studies as well as genetic selections. Moreover, using the Δ*pilB3-C3* knockout strains as well as hybrid pilins and flagellins constructed for this study, will shed light on the details of the initial interactions between pilins and PilB/C, a step in bacterial pilus biosynthesis that has long remained elusive.

Experimental procedures

Strains and growth conditions

The plasmids and strains used in this study are listed in Table S1. *H. volcanii* H53 and its derivatives were grown at 45°C in liquid or on solid agar (1.5% w/v) semi-defined Casamino Acid (CA) medium, supplemented with tryptophan and uracil (50 μg ml^{-1} final concentration) or complex Modified Growth Medium (MGM). Strains transformed with pTA963 are grown on CA medium supplemented with tryptophan (50 μg ml^{-1} final concentration) (Dyall-Smith, 2004). For selection of the deletion mutants (see below), 5-FOA was added at a final concentration of 150 μg ml^{-1} in CA medium, and uracil was added to a final concentration of 10 μg ml^{-1}. Strain H53 and the deletion mutants transformed with pTA963 or its derivatives 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}) (Blattner *et al*., 1977).

Generation of chromosomal deletions

Chromosomal deletions were generated by using a homologous recombination (pop-in pop-out) method previously described by Allers and Ngo (2003). Plasmid constructs were generated using overlap PCR as described previously by Hammelmann and Soppa (2008) and modified as described in Tripepi *et al*. (2010). To confirm the chromosomal replacement event occurred at the proper location on the chromosome, the genomic DNA isolated from colonies derived using these techniques was screened by PCR. The identities of the PCR products were verified by sequencing using the primers lying outside the gene of interest (primers used are listed in Table S2).

Surface adhesion assay

H. volcanii surface adhesion was assayed using a modified air-liquid interface (ALI) assay (O'Toole *et al*., 1999) as described in Esquivel *et al*. (2013). Briefly, 3 ml of culture in CA medium supplemented with tryptophan and/or uracil as necessary, at an optical density of 600 nm (OD_{600}) of ~ 0.3, was incubated in each well of a 12-well plate. Plastic coverslips (22 by 22 mm; 0.19 to 0.25 mm thick) were inserted into each well and incubated overnight at 45°C without shaking. Upon acetic acid fixing, coverslips were stained in 0.1% w/v crystal violet solution for 10 min. The coverslips were then washed with distilled water, air-dried and examined using light microscopy.

Motility assay

Motility assays were performed by stab-inoculating *H. volcanii* into motility plates containing 0.3% w/v agar as described in Tripepi *et al*. (2010) and incubated for 3 days at 45°C, unless otherwise noted.

Isolation and purification of surface filaments

The isolation of *H. volcanii* flagella or type IV pili supernatant fractions was performed by CsCl gradient purification as described previously by Fedorov (1994), with modifications as described in Tripepi *et al*. (2013). Briefly, to select for motile cells, colonies from a solid-agar plate were stab-inoculated into motility plates and cells from the outer motility ring, formed after 3 days, were inoculated into 5 ml CA liquid medium. Two litre of CA medium were inoculated with this 5-ml culture, and the cultures were harvested at an OD_{600} of approximately 0.3 by centrifugation at 8700 rpm (JA-10 rotor; Beckman) for 30 min. The supernatant was centrifuged again (8700 rpm for 30 min) and incubated at room temperature with 4% w/v polyethylene glycol (PEG) 6000 for 1 h. The PEG-precipitated proteins were then centrifuged at 16 000 rpm (JLA-16.250 rotor; Beckman) for 50 min at 4°C, and the surface filaments were purified by cesium chloride (CsCl) density gradient centrifugation (overnight centrifugation at 50 000 rpm) (VTI-65.1 rotor; Beckman). CsCl was dissolved in a 3 M NaCl saline solution to a final density of 1.37 g cm^{-1}.

Protein extraction, LDS-PAGE and Western blotting

Protein from cell pellets, TCA-precipitated supernatants, or surface filament containing CsCl fractions of *H. volcanii* strains were separated by LDS gel chromatography and stained by Coomassie or, in strains expressing His-tagged constructs, analysed by western blot using anti-His antibodies as described in Tripepi *et al*. (2012). Liquid cultures were grown until the early-log phase (OD_{600} ~ 0.3). Cells were collected by centrifugation at 4300 *g* for 10 min at 4°C. Cell pellets were resuspended and lysed in 1% v/v NuPAGE lithium dodecyl sulphate (LDS) supplemented with 50 mM dithiothreitol (DTT). The supernatants of relevant strains containing secreted proteins were precipitated with cold trichloroacetic acid (TCA) (10%, v/v), and pellets were washed twice with cold acetone (80%, v/v) and then resuspended in 1% LDS buffer supplemented with 50 mM DTT. The electrophoresis of protein samples was performed with 12% v/v Bis-Tris NuPAGE gels under denaturing conditions using morpholinepropanesulphonic acid (MOPS) buffer at pH 7.7. Proteins were transferred from the gel onto a polyvinylidene difluoride membrane using a Bio-Rad Transblot-SD semidry transfer cell at 15 V for 30 min. Western blots of whole-cell lysates of strains expressing C-terminally His-tagged constructs with a three amino acid linker sequence were probed with an anti-His antibody at a dilution of 1:1000, followed by a secondary anti-mouse antibody at a dilution of 1:10 000. Antibody-labelled protein bands were identified using the Amersham ECL Plus Western blotting detection system.

Electron microscopy

H. volcanii whole cells and CsCl gradient fractions were prepared as described in Tripepi *et al*. (2013). Cell cultures were fixed in CA medium with 2% v/v glutaraldehyde and 1% v/v paraformaldehyde for 1 h. Ten microlitres of the fixed culture was put onto glow-discharged copper grids coated with carbon-Formvar for 10 min. The grids were rinsed two times in ddH_2O and negatively stained using 1% w/v uranyl formate. Grids were then analysed using a Philips Tecnai 12 operating at 120 kV, and a 0Gatan US1000 2K × 2K camera. CsCl gradient density fractions were applied onto the glow

discharged grids and were left for 5 min at room temperature, washed with water, blotted with a filter paper, and stained with 2% w/v uranyl acetate for 10 s. Grids were then analysed using a Philips Tecnai 12 instrument operating at 120 kV and a Gatan US1000 2K-by-2 K (2024- by 2024-pixel) camera.

Acknowledgements

M.P. and R.E. were supported by National Aeronautics and Space Administration grant NNX10AR84G. R.E. was also supported by the 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, Friedhelm Pfeiffer, Michael Donnenberg, Ben Garcia, Fevzi Daldal and the Pohlschroder lab for helpful discussions.

References

Albers, S.V., and Pohlschroder, M. (2009) Diversity of archaeal type IV pilin-like structures. *Extremophiles* **13:** 403–410.

Albers, S.V., Szabo, Z., and Driessen, A.J. (2003) Archaeal homolog of bacterial type IV prepilin signal peptidases with broad substrate specificity. *J Bacteriol* **185:** 3918–3925.

Allers, T., and Ngo, H.P. (2003) Genetic analysis of homologous recombination in Archaea: *Haloferax volcanii* as a model organism. *Biochem Soc Trans* **31:** 706–710.

Bardy, S.L., and 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.

Blair, K.M., Turner, L., Winkelman, J.T., Berg, H.C., and Kearns, D.B. (2008) A molecular clutch disables flagella in the *Bacillus subtilis* biofilm. *Science* **320:** 1636–1638.

Blattner, F.R., Williams, B.G., Blechl, A.E., Denniston-Thompson, K., Faber, H.E., Furlong, L., *et al.* (1977) Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. *Science* **196:** 161–169.

Boyd, C.D., and O'Toole, G.A. (2012) Second messenger regulation of biofilm formation: breakthroughs in understanding c-di-GMP effector systems. *Annu Rev Cell Dev Biol* **28:** 439–462.

Craig, L., Volkmann, N., Arvai, A.S., Pique, M.E., Yeager, M., Egelman, E.H., and 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.

Dyall-Smith, M. (2004) The halohandbook – protocols for haloarchaeal genetics. [WWW document]. URL http://www.haloarchaea.com

Esquivel, R.N., Xu, R., and Pohlschroder, M. (2013) Novel archaeal adhesion pilins with a conserved N terminus. *J Bacteriol* **195:** 3808–3818.

Fedorov, O.V. (1994) Protofilament as a structural element of flagella of haloalkalophilic archaebacteria. *Can J Microbiol* **40:** 45–53.

Fröls, S., Ajon, M., Wagner, M., Teichmann, D., Zolghadr, B., Folea, M., *et al.* (2008) UV-inducible cellular aggregation of the hyperthermophilic archaeon *Sulfolobus solfataricus* is mediated by pili formation. *Mol Microbiol* **70:** 938–952.

Ghosh, A., and Albers, S.V. (2011) Assembly and function of the archaeal flagellum. *Biochem Soc Trans* **39:** 64–69.

Giltner, C.L., Nguyen, Y., and Burrows, L.L. (2012) Type IV pilin proteins: versatile molecular modules. *Microbiol Mol Biol Rev* **76:** 740–772.

Guttenplan, S.B., and Kearns, D.B. (2013) Regulation of flagellar motility during biofilm formation. *FEMS Microbiol Rev* **37:** 849–871.

Hammelmann, M., and Soppa, J. (2008) Optimized generation of vectors for the construction of *Haloferax volcanii* deletion mutants. *J Microbiol Methods* **75:** 201–204.

Hansen, S.K., Rainey, P.B., Haagensen, J.A., and Molin, S. (2007) Evolution of species interactions in a biofilm community. *Nature* **445:** 533–536.

Hartman, A.L., Norais, C., Badger, J.H., Delmas, S., Haldenby, S., Madupu, R., *et al.* (2010) The complete genome sequence of *Haloferax volcanii* DS2, a model archaeon. *PLoS ONE* **5:** e9605.

Haussler, S., and Fuqua, C. (2013) Biofilms 2012: new discoveries and significant wrinkles in a dynamic field. *J Bacteriol* **195:** 2947–2958.

Henche, A.L., Ghosh, A., Yu, X., Jeske, T., Egelman, E., and Albers, S.V. (2012) Structure and function of the adhesive type IV pilus of *Sulfolobus acidocaldarius*. *Environ Microbiol* **14:** 3188–3202.

Jarrell, K.F., and Albers, S.V. (2012) The archaellum: an old motility structure with a new name. *Trends Microbiol* **20:** 307–312.

Karatan, E., and Watnick, P. (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev* **73:** 310–347.

Kuchma, S., Kimberly, M.B., Judith, H.M., Nicole, T.L., Frederick, M.A., and O'Toole, G.A. (2007) BifA, a cyclic-di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *J Bacteriol* **189:** 8165–8178.

Large, A., Stamme, C., Lange, C., Duan, Z., Allers, T., Soppa, J., and Lund, P.A. (2007) Characterization of a tightly controlled promoter of the halophilic archaeon *Haloferax volcanii* and its use in the analysis of the essential *cct1* gene. *Mol Microbiol* **66:** 1092–1106.

Lassak, K., Ghosh, A., and Albers, S.V. (2012) 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., Peeters, E., Wrobel, S., and Albers, S.V. (2013) The one-component system ArnR: a membrane-bound activator of the crenarchaeal archaellum. *Mol Microbiol* **88:** 125–139.

McDougald, D., Rice, S.A., Barraud, N., Steinberg, P.D., and Kjelleberg, S. (2011) Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol* **10:** 39–50.

Monds, R.D., and O'Toole, G.A. (2009) The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol* **17:** 73–87.

Orell, A., Frols, S., and Albers, S.V. (2013a) Archaeal biofilms: the great unexplored. *Annu Rev Microbiol* **67:** 337–354.

Orell, A., Peeters, E., Vassen, V., Jachlewski, S., Schalles,

S., Siebers, B., and Albers, S.V. (2013b) Lrs14 transcriptional regulators influence biofilm formation and cell motility of Crenarchaea. *ISME J* **7:** 1886–1898.
O'Toole, G.A., and Kolter, R. (1998) Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Mol Microbiol* **30:** 295–304.
O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B., and Kolter, R. (1999) Genetic approaches to study of biofilms. *Methods Enzymol* **310:** 91–109.
Pohlschroder, M., Ghosh, A., Tripepi, M., and Albers, S.V. (2011) Archaeal type IV pilus-like structures – evolutionarily conserved prokaryotic surface organelles. *Curr Opin Microbiol* **14:** 357–363.
Reimann, J., Lassak, K., Khadouma, S., Ettema, T.J., Yang, N., Driessen, A.J., *et al*. (2012) Regulation of archaella expression by the FHA and von Willebrand domain-containing proteins ArnA and ArnB in *Sulfolobus acidocaldarius*. *Mol Microbiol* **86:** 24–36.
Szabó, Z., Stahl, A.O., Albers, S.V., Kissinger, J.C., Driessen, A.J., and Pohlschröder, M. (2007) Identification of diverse archaeal proteins with class III signal peptides cleaved by distinct archaeal prepilin peptidases. *J Bacteriol* **189:** 772–778.
Takhar, H.K., Kemp, K., Kim, M., Howell, P.L., and Burrows, L.L. (2013) The platform protein is essential for type IV pilus biogenesis. *J Biol Chem* **288:** 9721–9728.
Tripepi, M., Imam, S., and Pohlschroder, 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., Onder, O., Brisson, D., and Pohlschroder, 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.
Tripepi, M., Esquivel, R.N., Wirth, R., and Pohlschroder, M. (2013) *Haloferax volcanii* cells lacking the flagellin FlgA2 are hypermotile. *Microbiology* **159:** 2249–2258.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.