

# *Haloferax volcanii* Flagella Are Required for Motility but Are Not Involved in PibD-Dependent Surface Adhesion<sup>∇</sup>

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Although the genome of *Haloferax volcanii* contains genes (*flgA1-flgA2*) that encode flagellins and others that encode proteins involved in flagellar assembly, previous reports have concluded that *H. volcanii* is nonmotile. Contrary to these reports, we have now identified conditions under which *H. volcanii* is motile. Moreover, we have determined that an *H. volcanii* deletion mutant lacking flagellin genes is not motile. However, unlike flagella characterized in other prokaryotes, including other archaea, the *H. volcanii* flagella do not appear to play a significant role in surface adhesion. While flagella often play similar functional roles in bacteria and archaea, the processes involved in the biosynthesis of archaeal flagella do not resemble those involved in assembling bacterial flagella but, instead, are similar to those involved in producing bacterial type IV pili. Consistent with this observation, we have determined that, in addition to disrupting preflagellin processing, deleting *pibD*, which encodes the preflagellin peptidase, prevents the maturation of other *H. volcanii* type IV pilin-like proteins. Moreover, in addition to abolishing swimming motility, and unlike the *flgA1-flgA2* deletion, deleting *pibD* eliminates the ability of *H. volcanii* to adhere to a glass surface, indicating that a nonflagellar type IV pilus-like structure plays a critical role in *H. volcanii* surface adhesion.

To escape toxic conditions or to acquire new sources of nutrients, prokaryotes often depend on some form of motility. Swimming motility, a common means by which many bacteria move from one place to another, usually depends on flagellar rotation to propel cells through liquid medium (24, 26, 34). These motility structures are also critical for the effective attachment of bacteria to surfaces.

As in bacteria, rotating flagella are responsible for swimming motility in archaea, and recent studies suggest that archaea, like bacteria, also require flagella for efficient surface attachment (37, 58). However, in contrast to bacterial flagellar subunits, which are translocated via a specialized type III secretion apparatus, archaeal flagellin secretion and flagellum assembly resemble the processes used to translocate and assemble the subunits of bacterial type IV pili (34, 38, 54).

Type IV pili are typically composed of major pilins, the primary structural components of the pilus, and several minor pilin-like proteins that play important roles in pilus assembly or function (15, 17, 46). Pilin precursor proteins are transported across the cytoplasmic membrane via the Sec translocation pathway (7, 20). Most Sec substrates contain either a class I or a class II signal peptide that is cleaved at a recognition site that lies subsequent to the hydrophobic portion of the signal peptide (18, 43). However, the precursors of type IV pilins contain class III signal peptides, which are processed at recognition sites that precede the hydrophobic domain by a prepilin-specific peptidase (SPase III) (38, 43, 45). Similarly, archaeal flagellin precursors contain a class III signal peptide that is processed by a prepilin-specific peptidase homolog (FlaK/

PibD) (3, 8, 10, 11). Moreover, flagellar assembly involves homologs of components involved in the biosynthesis of bacterial type IV pili, including FlaI, an ATPase homologous to PilB, and FlaJ, a multispreading membrane protein that may provide a platform for flagellar assembly, similar to the proposed role for PilC in pilus assembly (38, 44, 53, 54). These genes, as well as a number of others that encode proteins often required for either flagellar assembly or function (*flaCDEFG* and *flaH*), are frequently coregulated with the *flg* genes (11, 26, 44, 54).

Interestingly, most sequenced archaeal genomes also contain diverse sets of genes that encode type IV pilin-like proteins with little or no homology to archaeal flagellins (3, 39, 52). While often coregulated with *pilB* and *pilC* homologs, these genes are never found in clusters containing the motility-specific *flaCDEFG* and *flaH* homologs; however, the proteins they encode do contain class III signal peptides (52). Several of these proteins have been shown to be processed by an SPase III (4, 52). Moreover, in *Sulfolobus solfataricus* and *Methanococcus maripaludis*, some of these archaeal type IV pilin-like proteins were confirmed to form surface filaments that are distinct from the flagella (21, 22, 56). These findings strongly suggest that the genes encode subunits of pilus-like surface structures that are involved in functions other than swimming motility.

In bacteria, type IV pili are multifunctional filamentous protein complexes that, in addition to facilitating twitching motility, mediate adherence to abiotic surfaces and make close intercellular associations possible (15, 17, 46). For instance, mating between *Escherichia coli* in liquid medium has been shown to require type IV pili (often referred to as thin sex pili), which bring cells into close proximity (29, 30, 57). Recent work has shown that the *S. solfataricus* pilus, Ups, is required not only for efficient adhesion to surfaces of these crenarchaeal

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cells but also for UV-induced aggregation (21, 22, 58). Frols et al. postulate that autoaggregation is required for DNA exchange under these highly mutagenic conditions (22). *Halobacterium salinarum* has also been shown to form  $Ca^{2+}$ -induced aggregates (27, 28). Furthermore, conjugation has been observed in *H. volcanii*, which likely requires that cells be held in close proximity for a sustained period to allow time for the cells to construct the cytoplasmic bridges that facilitate DNA transfer between them (35).

To determine the roles played by haloarchaeal flagella and other putative type IV pilus-like structures in swimming and surface motility, surface adhesion, autoaggregation, and conjugation, we constructed and characterized two mutant strains of *H. volcanii*, one lacking the genes that encode the flagellins and the other lacking *pibD*. Our analyses indicate that although this archaeon was previously thought to be nonmotile (14, 36), wild-type (wt) *H. volcanii* can swim in a flagellum-dependent manner. Consistent with the involvement of PibD in processing flagellins, the peptidase mutant is nonmotile. Unlike nonhalophilic archaea, however, the flagellum mutant can adhere to glass as effectively as the wild type. Conversely, the  $\Delta pibD$  strain fails to adhere to glass surfaces, strongly suggesting that in *H. volcanii* surface adhesion involves nonflagellar, type IV pilus-like structures.

#### MATERIALS AND 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. An ECL Plus Western blotting system, horseradish peroxidase-linked sheep anti-mouse antibody, and donkey anti-rabbit antibody were purchased from Amersham Biosciences. Polyvinylidene difluoride membrane was 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, and Company. 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.** Unless noted otherwise, all *H. volcanii* strains (Table 1) were grown at 45°C in complex medium (CX) or defined liquid medium (CDM or CA medium) or on solid medium containing agar (19, 40). The CX (complex medium) contains the following (per liter): 125 g of NaCl, 45 g of  $MgCl_2 \cdot 6H_2O$ , 10 g of  $MgSO_4 \cdot 7H_2O$ , 10 g of KCl, 1.34 g of  $CaCl_2 \cdot 2H_2O$ , 3 g of yeast extract, and 5 g of tryptone (19). For high- and low-salt CX preparations, total salt concentrations (proportional amounts of each salt) were 23% and 14%, respectively, compared to the 19% total salt concentration of the CX described above. CA medium contains the following (per liter): 5 g of Casamino acids, 2.4 ml of 1 M KOH, 600 ml of 30% salt water solution (described below), 6 ml of 0.5 M  $CaCl_2$ , 900  $\mu$ l of thiamine and biotin solution (9.6 ml of thiamine [1 mg/ml], 1.2 ml of biotin [1 mg/ml] in 100 ml). Salt water solution (30%) contains the following (per liter): 240 g of NaCl, 30 g of  $MgCl_2 \cdot 6H_2O$ , 35 g of  $MgSO_4 \cdot 7H_2O$ , 7 g of KCl, 5 ml of 1 M  $CaCl_2 \cdot 2H_2O$ , 2 ml of 1 M Tris-HCl buffer (pH 7.5) (40). CDM contains the following (per liter): 125 g of NaCl, 50 g of  $MgCl_2 \cdot 6H_2O$ , 5 g of  $K_2SO_4$ , 0.26 g of  $CaCl_2$ , 5 ml of 1 M  $NH_4Cl$ , 22.5 ml of Na succinate (10g/100 ml of double-distilled  $H_2O$  [dd $H_2O$ ]), 2.5 ml of 10% glycerol, 2 ml of 0.5 M  $K_2HPO_4$  (8.7g/100 ml of dd $H_2O$ ), 1 ml of trace elements (36 mg of  $MnCl_2 \cdot 4H_2O$ , 44 mg of  $ZnSO_4 \cdot 7H_2O$ , 230 mg of  $FeSO_4 \cdot 7H_2O$ , and 5 mg of  $CuSO_4 \cdot 5H_2O$  per 100 ml of dd $H_2O$ ), 0.8 ml of 1 mg/ml thiamine, 0.1 ml of 1 mg/ml biotin (19). Fifteen grams of agar was added for regular plates, and 3 g of agar was added for motility plates. To ensure equal concentration of agar in all plates, the agar was completely dissolved prior to autoclaving, and the autoclaved medium was stirred before the plates were poured. *H. volcanii* strain H53 (6) was grown in CDM and CA medium supplemented with tryptophan and uracil (50  $\mu$ g/ml final concentration). *H. volcanii* strain H98 (6) was grown in CX supplemented with thymidine (40  $\mu$ g/ml) or in CDM and CA medium supplemented with thymidine, hypoxanthine (40  $\mu$ g/ml), and uracil (50  $\mu$ g/ml, unless noted differently). 5-FOA,

TABLE 1. Strains and plasmids

Plasmid or strain	Relevant properties or description	Reference or source
<b>Plasmids</b>		
pTA131	Ampr; pBluescript II with BamHI-XbaI fragment from pGB70 containing <i>pdfx-pyrE2</i>	6
pRV1- <i>ptna</i>	Amp <sup>r</sup> Nov <sup>r</sup> <i>ptna</i>	31
pMT1	pTA131 containing chromosomal <i>flgA1-flgA2</i> region	This study
pMT2	pTA131 containing chromosomal <i>pibD</i> region	This study
pMT3	pRV1- <i>ptna</i> containing <i>flgA1-flgA2</i> -His	This study
pMT4	pRV1- <i>ptna</i> containing <i>pibD</i> -His	This study
pMT5	pRV1- <i>ptna</i> containing Hvo_A0632-His; type IV pilin-like gene	This study
pMT6	pRV1- <i>ptna</i> containing Hvo_2451-His; type IV pilin-like gene	This study
<b>Strains</b>		
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> 80dlacZ $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>recA1 endA hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) <i>supE44 thi-1 gyrA relA1</i>	Invitrogen
DL739	MC4100 <i>recA dam-13::Tn9</i>	13
<i>H. volcanii</i>		
H98	$\Delta$ <i>pyrE2</i> $\Delta$ <i>hdrB</i>	6
H53	$\Delta$ <i>pyrE2</i> $\Delta$ <i>trp</i>	6
MT1	H98 $\Delta$ <i>flgA1</i> $\Delta$ <i>flgA2</i>	This study
MT2	H53 $\Delta$ <i>flgA1</i> $\Delta$ <i>flgA2</i>	This study
MT3	H98 $\Delta$ <i>pibD</i>	This study
MT4	H53 $\Delta$ <i>pibD</i>	This study
MT5	H98 containing pMT3	This study
MT6	H98 containing pMT4	This study
MT7	MT3 containing pMT3	This study
MT8	MT3 containing pMT4	This study
MT9	H98 containing pMT5	This study
MT10	H98 containing pMT6	This study
MT11	MT3 containing pMT5	This study
MT12	MT3 containing pMT6	This study
<i>aglB</i> strain	H53 $\Delta$ <i>aglB</i>	1

for the selection of  $\Delta$ *flgA1*  $\Delta$ *flgA2* and  $\Delta$ *pibD* deletion mutants (see below), was added at a final concentration of 150  $\mu$ g/ml. *H. volcanii* strains transformed with pRV1-*ptna* (31) and its derivatives were grown in medium supplemented with novobiocin (final concentration, 2  $\mu$ g/ml). To induce protein expression from the pRV10-*ptna* plasmid, tryptophan (final concentration, 100  $\mu$ g/ml) was added to the medium. *E. coli* strains (Table 1) were grown at 37°C in NZCYM medium (Fisher; 22 g NZCYM per 1 liter of dd $H_2O$ ), supplemented with ampicillin (200  $\mu$ g/ml) when necessary (12).

**Assembly of knockout constructs.** Plasmid constructs for use in homologous recombination were generated as previously described (5). In brief, approximately 700 nucleotides flanking each end of the gene of interest were PCR amplified (primers are listed in Table 2). The amplified flanking regions were sequentially cloned into the haloarchaeal suicide vector pTA131. First, the upstream flanking region was cloned into pTA131 digested with restriction enzymes EcoRI and HindIII. The construct was then digested with BamHI and XbaI, and the downstream flanking region was inserted. The final constructs, pMT1 and pMT2, contain upstream and downstream region inserts that are identical to the corresponding chromosomal regions of *flgA1-flgA2* and *pibD*, respectively.

**Generating chromosomal deletions.** Chromosomal deletions were generated using the homologous recombination (pop-in-pop-out) method as previously described (5). The pMT1 and pMT2 constructs were isolated from *E. coli* DH5 $\alpha$  and transformed into *E. coli* DL739 (13). Using the standard polyethylene glycol method, nonmethylated plasmid DNA isolated from *E. coli* DL739 was used to transform *H. volcanii* strains H98 and H53 (6). A single homologous recombination event between one of the flanking regions cloned into the plasmid and the

Primer name

Sequences (5'–3')<sup>a</sup>

Target sequence

TABLE 2. Primers used for PCR amplification

Primer name	Sequences (5'–3') <sup>a</sup>	Target sequence
FigA1/2KO up for HindIII	GCCGTA <sup>u</sup> AAAAGCTTGTGCTCGTCTCCGACTC	705 bp upstream of <i>figA1</i> start codon, extension toward gene
FigA1/2KO up rev EcoRI	TTA <sup>u</sup> CTTGGAAATTCGTTAACCCCGCACCCGA	1 bp upstream of <i>figA1</i> start codon, extension away from gene
FigA1/2KOD for BamHI	ACGTCGGGATCCAGATTTTCGTGGGTTT	1 bp downstream of <i>figA12</i> stop codon, extension away from gene
FigA1/2KOD rev XbaI	GCGACGCTTAGAACCGTTCCTCCTCGTC	705 bp downstream of <i>figA12</i> stop codon extension toward gene
PibDKOup for HindIII	TTATTTAAGCTTCTCTCGCGCCGGGATC	695 bp upstream of <i>pibD</i> start codon, extension toward gene
PibDKOup rev EcoRI	GCAGTGAATTTCTACCCCGGAGACTGGGG	1 bp upstream of <i>pibD</i> start codon, extension away from gene
PibDKOD for BamHI	ATTATGGATCCGTCGCTGCGGAGTCCG	1 bp downstream of <i>pibD</i> stop codon, extension away from gene
PibDKOD for XbaI	GGATAGTCTAGAGTCCGTCGCGGGGCTC	705 bp downstream of <i>pibD</i> stop codon extension toward gene
FigA1/2for	CGCCCGTGGGAGAGGTGATT	71 bp downstream of <i>figA1</i> start codon, extension toward gene
FigA1/2rev	CGAGGCGGACCCGTCAGGAAA	262 bp upstream of <i>figA12</i> stop codon, extension toward gene
PibDfor	TTCGCCTTCGGACTGCTCGGT	283 upstream of <i>pibD</i> start codon, extension toward gene
PibDrev	CGCGACGAACTCTGGGAGGAAG	255 downstream of <i>pibD</i> stop codon, extension toward gene
FigA1for NdeI	ATTAATCATATGTTTCGAAAACATCAACGAA	1 bp downstream of <i>figA1</i> start codon, extension toward gene
FigA2 6-His EcoRI rev	TATATTTGAATTTCTCAGTGTGATGGTGTGAT	1 bp upstream of <i>figA2</i> stop codon, extension toward gene
PibDfor NdeI	GCGGGCCCGCCAGTTCCGACGGGGTT	1 bp downstream of <i>pibD</i> start codon, extension toward gene
PibD 6-His EcoRI rev	GCTATCCATATATGTTCCGGTATCCGCCAGC	1 bp upstream of <i>pibD</i> stop codon, extension toward gene
Hwo_A0632 forNdeI	TATATTTGAATTTCTCAGTGTGATGGTGTGAT	1 bp downstream of Hwo_A0632 start codon, extension toward gene
Hwo_A0632 6-His EcoRI rev	CCCGCGCATATGATATCAAGAAATTCCTTAG	1 bp upstream of Hwo_A0632 stop
Hwo_2451 for NdeI	TATATTTGAATTTCTCAGTGTGATGGTGTGAT	1 bp downstream of Hwo_2451 start codon, extension toward gene
Hwo_2451 6-His EcoRI rev	GCGGGCCCGCCGTCGCGTGTACGGTTTG AATTAACCATATGCAACTCAAAACATCTCTTCAC GTAATTTGAATTTCTCAGTGTGATGGTGTGAT TGCGGGCCCGCTCCGTTGTAGGTCCA	1 bp upstream of Hwo_2451 stop

<sup>a</sup> Restriction endonuclease sites are underlined.

chromosome (pop in) was selected for by growth on CA agar lacking uracil that was supplemented with either thymidine and hypoxanthine or tryptophan, depending on the strain. pMT1 and pMT2 contain *pyrE2*, a gene that catalyzes the biosynthesis of uracil, which allows positive selection for recombination events (Table 1). PCR was used to confirm that recombination occurred at the proper location on the chromosome. Recombinants were grown for 48 h in liquid CA medium supplemented with thymidine, hypoxanthine, and uracil (H98) or with tryptophan and uracil (H53) to allow a second recombination event, which results in excision of the plasmid from the chromosome (pop out). Plasmid excision results in either reversion to the parental locus or replacement of the chromosomal gene with the insert. After 48 h, H98 liquid cultures were transferred to CA agar plates supplemented with thymidine, hypoxanthine, uracil (10  $\mu\text{g/ml}$ ), and 5-FOA while H53 strains were transferred to CA agar plates supplemented with tryptophan, uracil (10  $\mu\text{g/ml}$ ), and 5-FOA. These growth conditions permit the growth only of cells in which the plasmid has been excised from the chromosome. Colonies derived from these cultures were screened by PCR to confirm the chromosomal replacement event (primers used are listed in Table 2). The correct identities of the PCR products were confirmed by sequencing using the same primers. The  $\Delta\text{flgA1 } \Delta\text{flgA2}$  deletion mutants generated in strains H98 and H53 were designated MT1 and MT2, respectively, and the *pibD* deletion mutants generated in strains H98 and H53 were designated MT3 and MT4, respectively (Table 1).

**Construction of expression vectors, expression and extraction of proteins, and Western blotting.** To investigate protein expression, we created gene fusions that encode proteins containing a C-terminal His tag; the inducible tryptophan promoter (*tna*) drives expression of these gene fusions (31). Genes were amplified using the primers listed in Table 2 and inserted into pRV1-*ptna* digested with NdeI and EcoRI. pMT3, pMT4, pMT5, and pMT6 (pRV1-*ptna* containing C-terminally His-tagged FlgA2, PibD, Hvo\_A0632, and Hvo\_02451, respectively) were isolated from *E. coli* DH5 $\alpha$  and transformed into *E. coli* DL739 (Table 1). Using the standard polyethylene glycol method, nonmethylated plasmid DNA isolated from *E. coli* DL739 was used to transform *H. volcanii* strains (19). Tryptophan was added to the CX to a final concentration of 100  $\mu\text{g/ml}$  to induce gene expression from these plasmids.

All protein samples were stored in NuPAGE lithium dodecyl sulfate sample buffer, supplemented with 50 mM dithiothreitol. Samples were run on Bis-Tris NuPAGE gels (Invitrogen) under denaturing conditions using morpholinepropanesulfonic acid (MOPS). Proteins were transferred from the gel to 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 from *H. volcanii* strains expressing His-tagged constructs were probed with anti-His antibody at a dilution of 1:1,000, followed by a secondary anti-mouse antibody at a dilution of 1:10,000. Cell lysates isolated from wild-type or  $\Delta\text{pibD } H. volcanii$  were also probed with anti-*H. volcanii* S-layer (cell wall) glycoprotein (anti-CWG) antibody at a dilution of 1:1,000 followed by a secondary anti-rabbit antibody at a dilution of 1:10,000 (23). Antibody-labeled protein bands were identified using an Amersham ECL Plus Western blotting detection system.

**Surface adhesion assay.** Surface adhesion was assayed using a modified air-liquid interface (ALI) assay protocol (42) as follows: 3 ml of culture in CX at an optical density at 600 nm ( $\text{OD}_{600}$ ) of  $\sim 0.3$ , was incubated in each well of a 12-well plate. Glass coverslips (22 by 22 mm; 0.19 to 0.25 mm thick) were inserted into each well at an angle of 90°. Lids were placed over the wells; the plates were then placed on wet paper towels and wrapped in polyvinyl paper to limit evaporation. The wrapped plates were incubated at 45°C with or without shaking. After various time intervals, coverslips were removed from the wells with forceps, submerged for 3 min in 2% acetic acid, and then allowed to air dry. When the coverslips were completely dry, they were stained in 0.1% crystal violet for 10 min. The coverslips were then washed three times with distilled water. Stained coverslips were air dried and then examined using light microscopy.

**Aggregation assays.** To identify conditions under which *H. volcanii* can aggregate, 3 ml of *H. volcanii* cultures grown in CX or CA medium to an  $\text{OD}_{600}$  of  $\sim 0.3$  were incubated at 45°C in each well of a 12-well plate. To test the effect of calcium on cell aggregation, various concentrations of  $\text{CaCl}_2$  were added to the cultures. To test the effect of magnesium,  $\text{MgCl}_2$  was added to the cultures. CA medium was also incubated with  $\text{CaCl}_2$  to control for nonspecific precipitation of calcium. Twelve-well plates were examined for cell aggregates at various time intervals.

**Motility assays.** The motility assay was performed in 0.3% motility agar plates of CX, CA medium, or CDM. Agar media were supplemented with tryptophan, thymidine, hypoxanthine, uracil, and novobiocin, depending on the *H. volcanii* strain being assayed. A toothpick was used to stab inoculate the agar.

**Conjugation assay.** Conjugation was assayed using a modified version of a protocol previously described by Mevarech and Werczberger (35). In brief, equal volumes (1 to 5 ml) from cultures of two different auxotrophic *H. volcanii* strains (H53 and H98) at  $\text{OD}_{600}$ s of 0.5 to 0.9 were mixed and filtered through Millipore Swinex 25 filter units with Millipore 0.45- $\mu\text{m}$ -pore-size type HA 25-mm filters using a 5-ml syringe. The filter unit was then disassembled, and filter discs were placed cell side up on CX supplemented with thymidine and incubated at 45°C overnight. After incubation, the filters were removed from the medium, placed in 2-ml Eppendorf tubes containing 1 ml of 18% salt water (19), and shaken on a rotator for 1 h. An undiluted sample and a sample that was diluted 1:100 were plated on CA agar medium supplemented with uracil. Twenty microliters was also plated on CX agar supplemented with thymidine for viable cell counts.

A liquid conjugation assay was performed as follows: 1 ml from each H53 and H98 culture at equal cell density was mixed; 3 ml of CX supplemented with thymidine was added to the mix, and the mixed cultures were grown overnight at 45°C in a shaking incubator. After the incubation, 2 ml from the mixed cultures was centrifuged, and pellets were resuspended in 100  $\mu\text{l}$  of CA medium, which was then plated on CA agar supplemented with uracil. Conjugation assays using H98 and H53 containing the *flgA1-flgA2* or *pibD* deletions were carried out the same way. Conjugation frequencies were expressed as the number of transconjugants per CFU of the donor. The reported results are the mean values from three separate experiments.

## RESULTS

***H. volcanii* exhibits swimming motility.** Although the current description of *H. volcanii* notes that this haloarchaeon is nonmotile (14), *in silico* analyses of the *H. volcanii* genome have identified homologs of genes that encode flagellins in other archaea, as well as genes that encode proteins involved in archaeal flagellum biosynthesis (25, 47, 49) (Fig. 1A). Moreover, with the exception of the gene encoding the SPase III, these genes are clustered with genes that encode proteins involved in chemotaxis, reminiscent of the organization of a similar *fla-che* cluster of genes in *Halobacterium salinarum* sp. R1 (48) (Fig. 1A). Similar to other reported archaeal flagellins, the *H. volcanii* FlgA1 and FlgA2 flagellin homologs contain a predicted class III Sec signal peptide and several predicted N-glycosylation sites (33, 38, 54) (Fig. 1B).

Here, we show that *H. volcanii* produces a growth halo after stab inoculation in a complex 0.3% agar motility medium (Fig. 2A). Consistent with microarray data, which indicate that genes in this *fla* cluster are expressed at low levels in a defined medium (CDM) relative to the expression levels observed in the CX (C. Daniels, personal communication), *H. volcanii* is nonmotile in the CDM (Fig. 2A). However, we have identified a distinct defined medium (CA medium) in which *H. volcanii* is motile (see Materials and Methods for medium composition) (Table 3 and Fig. 2A). The growth halos observed on CA motility plates are smaller than those on CX plates, likely reflecting the longer doubling time for *H. volcanii* grown in the CA medium (Table 3 and Fig. 2A).

To confirm that *H. volcanii* swimming motility requires functional flagella, we used homologous recombination to construct a strain in which *flgA1* and *flgA2* are deleted. The deletion was verified using PCR. In PCRs that included primers designed to amplify adjacent upstream and downstream regions, including the *flgA1-flgA2* genes, the PCR products generated for the deletion strain were smaller than those obtained for the parent strain, as expected (Fig. 3A).

We determined that the *H. volcanii*  $\Delta\text{flgA1 } \Delta\text{flgA2}$  strain is not motile in CX-0.3% agar medium (Fig. 2B). To confirm that this phenotype is due to the absence of flagellins, the deletion strain was complemented with a plasmid that ex-



TABLE 3. Phenotypic characterization of *H. volcanii*  $\Delta flgA1$   $\Delta flgA2$  and  $\Delta pibD$  deletion strains

Strain <sup>a</sup>	Value for the characteristic on the indicated medium											
	Minimal doubling time (h) <sup>b,c</sup>		Competition assay (H53/H98)		Conjugation frequency		Ca <sup>2+</sup> -dependent aggregation <sup>b</sup>		Motility (halo diam [cm] after 72 h) <sup>b,d</sup>		Adhesion <sup>b</sup>	
	CX	CA	CX	CA	CA + filter	Liquid CA	CX	CA	CX	CA	CX	CA
WT	3.5	4.5	50/50	50/50	10 <sup>-4</sup>	10 <sup>-8</sup>	-	+	0.9	0.7	+	+
$\Delta flgA1$ $\Delta flgA2$ strain	3.5	4.5	50/50	50/50	10 <sup>-4</sup>	10 <sup>-8</sup>	-	+	-	-	+	+
$\Delta pibD$ strain	3.5	4.5	50/50	50/50	10 <sup>-4</sup>	10 <sup>-8</sup>	-	+	-	-	-	-

<sup>a</sup> Both the H53 and H98 backgrounds were used for all strains.

<sup>b</sup> No differences were observed whether H98 or H53 background was used.

<sup>c</sup> Standard deviation, 0.5 h.

<sup>d</sup> Standard deviation, 0.22 and 0.29 cm in CX and CA motility media, respectively.

as the recent report suggesting that *Pyrococcus furiosus* (37) and *S. solfataricus* (22, 58) flagella are involved in biofilm formation and autoaggregation, we attempted to determine whether our flagellin deletion mutants have phenotypes similar to those of bacterial type IV pili and archaeal flagellum deletion mutants. We first determined that the *H. volcanii* flagellin mutants have no obvious growth defects. Under standard laboratory growth conditions, we found that the flagellin deletion mutant grows as well as the wild type in either complex or defined medium (Table 3). The  $\Delta flgA1$   $\Delta flgA2$  deletion strain also grows as well as the wild-type in CX containing a high (23%) or low (14%) salt concentration (5.5- and 6-h minimal doubling times, respectively). Moreover, similar growth rates were observed in CX at high (48°C) and low (30°C) temperatures (4- and 8-h minimal doubling times, respectively).

Since bacterial type IV pili are involved in conjugation (29, 30, 57), we tested whether the *H. volcanii* conjugation also requires the type IV pilus-like flagella by comparing the rate of conjugation between the H53 ( $\Delta trp$   $\Delta pyrE2$ ) and H98 ( $\Delta hdrB$   $\Delta pyrE2$ ) parental strains to the conjugation rates for the  $\Delta flgA1$   $\Delta flgA2$  deletion mutants of the corresponding strains, allowing us to identify both the dominant and recessive effects of the

$\Delta flgA1$   $\Delta flgA2$  mutation on conjugation. Upon confirming that the flagellin deletion mutants grew as well as the wild type (Table 3), we used the conjugation assay described by Mevarech and Werczberger to investigate the effect of the flagellin gene deletions on conjugation (35). We observed that the rates of conjugation between the modified  $\Delta flgA1$   $\Delta flgA2$  strains are similar to the rates between the parental strains when the strains are cocultured on a filter placed on a complex medium (CX) plate and subsequently cocultured in minimal medium (CA medium) lacking tryptophan and thymidine (conjugation frequency, 10<sup>-4</sup>) (Table 3). This result suggests that flagella are not involved in *H. volcanii* conjugation when cells are grown in close proximity. As the thin pilus of *E. coli* is required only for conjugation in liquid (29), we developed a liquid conjugation assay, which to the best of our knowledge is the first reported use of this type of assay in an archaeon. Although the frequency was significantly lower (10<sup>-8</sup>), we determined that conjugation does occur between cocultured *H. volcanii* auxotrophic strains. Interestingly, the conjugation frequency for the flagellin deletion mutants is similar to the frequency determined for the auxotrophic parent strains (Table 3).

Kawakami et al. have reported that *H. volcanii* can aggregate in a Ca<sup>2+</sup>-dependent manner, albeit with low efficiency (28). We determined that *H. volcanii* Ca<sup>2+</sup>-induced aggregation is highly efficient when cells are grown in CA medium but not in CX (Fig. 5). This aggregation was not observed when the divalent cation Mg<sup>2+</sup> is added to the cultures or when CA medium was incubated with CaCl<sub>2</sub> (Fig. 5). To determine whether this Ca<sup>2+</sup>-induced *H. volcanii* aggregation is flagellum-dependent, we compared aggregation of the H98 and the  $\Delta flgA1$   $\Delta flgA2$  strains using the modified aggregation protocol and determined that both strains aggregate in a Ca<sup>2+</sup>-dependent manner within a few minutes (Table 3).

Finally, to determine whether *H. volcanii* surface adhesion involves flagella, we used an air-liquid interface (ALI) assay modified from O'Toole et al. (42) (see Materials and Methods) to compare the ability of the parent and mutant strains to adhere to glass coverslips. These experiments were performed in cultures grown with shaking and cultures grown without shaking as *S. solfataricus* surface adhesion was flagellum dependent only in shaking cultures (58; also V. Albers, personal communication). Crystal violet staining of *H. volcanii* adhering to the air-liquid interface indicated that both the *H. volcanii* wild-type and the  $\Delta flgA1$   $\Delta flgA2$  strains adhere efficiently to

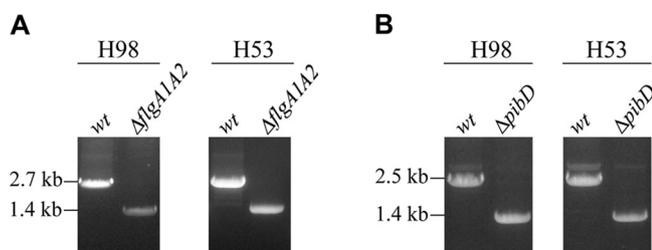


FIG. 3. Disruption of the chromosomal *flgA1-flgA2* and *pibD* loci in *H. volcanii* strains H98 and H53. PCR amplification was performed using primers against the flanking regions. (A) Amplification of approximately 700 nucleotides upstream and 700 bp downstream of *flgA1-flgA2*, using template DNA isolated from the wild-type (H98 or H53) or the  $\Delta flgA1$   $\Delta flgA2$  *H. volcanii* strains (MT1 or MT2, respectively). (B) Amplification of approximately 700 nucleotides upstream and 700 bp downstream of *pibD*, using template DNA isolated from the wild-type (H98 and H53) or the  $\Delta pibD$  *H. volcanii* strains (MT3 or MT4, respectively). As expected, the amplicons are approximately 1,300 and 1,100 nucleotides smaller for the  $\Delta flgA1$   $\Delta flgA2$  and  $\Delta pibD$  deletion strains, respectively, than the amplicons obtained using DNA isolated from the parental strains.

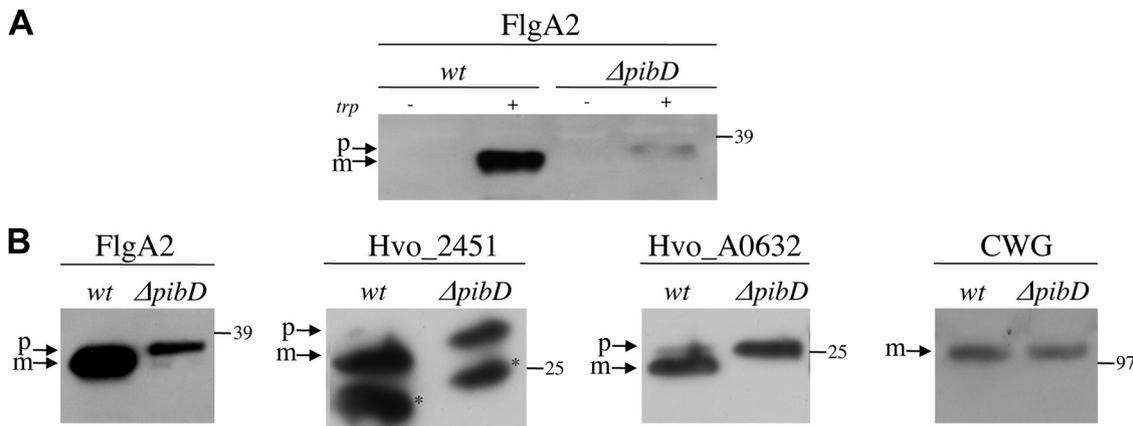


FIG. 4. *H. volcanii* PibD processes class III signal peptide-containing preproteins, including flagellins and other pilin-like proteins. (A) *H. volcanii* H98 (wt) and MT3 ( $\Delta pibD$ ) strains expressing FlgA1/FlgA2-His were grown to mid-log phase in CA medium in which the promoter was turned on and off by the addition of tryptophan. (B) *H. volcanii* H98 (wt) and MT3 ( $\Delta pibD$ ) strains expressing FlgA1/FlgA2-His, Hvo\_A0632-His, or Hvo\_02451-His in a tryptophan-inducible manner were grown to mid-log phase in CX plus tryptophan. Equivalent amounts of whole-cell lysates were subjected to SDS-PAGE and visualized by immunoblotting using an anti-His antibody. Cell extracts of H98 (wt) and  $\Delta pibD$  strains expressing C-terminally His-tagged Hvo\_A0632 were also tested for S-layer (cell-wall) glycoprotein (CWG) migration by immunoblotting using an anti-CWG antibody (23). Predicted positions of precursor (p) and mature (m) proteins are indicated. The lower bands in the Hvo\_2451 lanes, indicated by an asterisk, are likely degradation products of Hvo\_2451 precursor and mature proteins. The migration of molecular mass standards (expressed in kilodaltons) is indicated on the right.

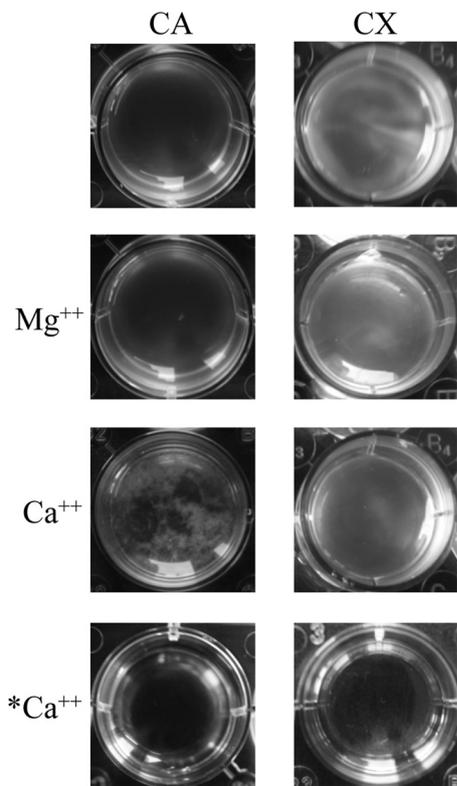


FIG. 5. *H. volcanii* Ca<sup>2+</sup>-induced aggregation in a defined medium. Adding 10 mM Ca<sup>2+</sup> induced aggregation of *H. volcanii* (H98) in mid-log phase cultures. While the majority of cells aggregated within 20 min after Ca<sup>2+</sup> addition in defined medium (CA), aggregation was not apparent when Mg<sup>2+</sup> was added to the cultures or in complex medium (CX). No aggregation was observed when Ca<sup>2+</sup> was added to medium lacking *H. volcanii* (\*Ca<sup>2+</sup>).

glass at the air-liquid interface whether the cells are shaken or not (Fig. 6). Our results, for what we believe is the first description of an assay demonstrating haloarchaeal surface adhesion, suggest that surface structures other than flagella are involved in *H. volcanii* conjugation, surface adhesion, and Ca<sup>2+</sup>-induced aggregation.

**The *H. volcanii* PibD homolog is required for processing a flagellin precursor, as well as precursors of other pilin-like proteins.** In *M. maripaludis*, FlaK, an SPase III, specifically processes preflagellins, while EppA, a distinct SPase III, processes the precursors of its pilins, all of which belong to a specific subgroup of type IV pilins (9, 52). Conversely, the *S. solfataricus* SPase III, PibD, has broader substrate specificity, processing both preflagellins and prepilins (4, 9). Like the genomes of the *Sulfolobales*, haloarchaeal genomes sequenced to date encode only a single SPase III and contain a number of predicted type IV pilins (52). To confirm that the *H. volcanii* SPase III cleaves preflagellins and to determine whether this proposed PibD homolog also cleaves the precursors of type IV pilin-like proteins, *H. volcanii* strain H98 containing a *pibD* deletion was constructed (MT3). The *pibD* deletion in this strain was confirmed in accordance with the methods used to confirm the  $\Delta flgA1$   $\Delta flgA2$  deletions (see above) (Fig. 3B). We then transformed H98 (wt) and MT3 with a pRV1-*ptna* plasmid that expresses FlgA1/FlgA2-His when cells are grown in the presence of tryptophan (Table 1, pMT3). Western blot analyses showed that protein fractions isolated from the transformed H98 strain cultured under inducing conditions contained a band migrating faster than the band observed in fractions isolated from the corresponding  $\Delta pibD$  transformant (Fig. 4A). MT3 transformants grown in CX also displayed a second band that migrated faster than the principal band, possibly representing a degradation product. In fact, in both CA medium and CX, the His-tagged FlgA2 observed in MT3

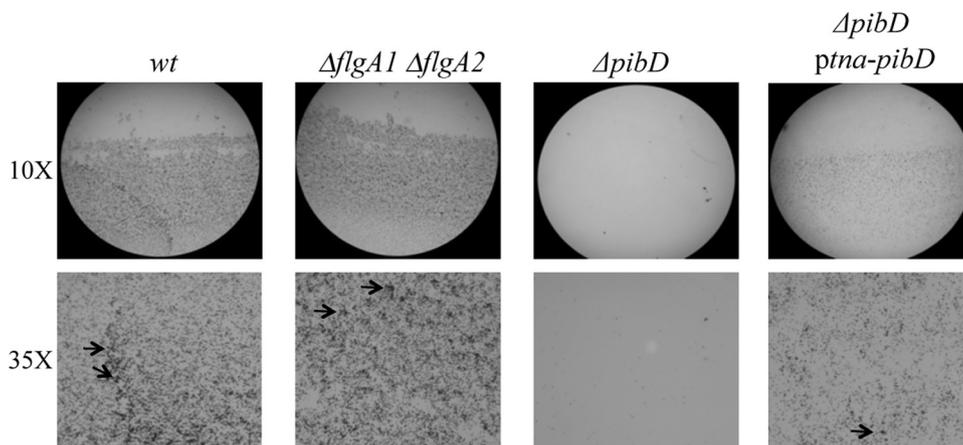


FIG. 6. *H. volcanii* adheres to glass in a FlgA1/FlgA2-independent, PibD-dependent manner. Adhesion to glass coverslips was tested using a modified ALI assay (42). Light micrographs of the coverslips taken at  $\times 10$  and  $\times 35$  magnifications are shown. Coverslips were placed in individual wells of 12-well plates, each containing 3 ml of a mid-log phase *H. volcanii* liquid culture (H98, wt, MT1,  $\Delta flgA1 \Delta flgA2$ ; MT3,  $\Delta pibD$ ; and MT7,  $\Delta pibD ptna-pibD$ ). After overnight incubation, cells were fixed with 2% acetic acid, stained with 0.1% crystal violet, and observed by light microscopy. Attachment of H98 and MT1 to coverslips was observed within 10 min of incubation (data not shown). Multicellular structures reminiscent of microcolonies (a subset indicated by arrows) were observed.

was significantly less abundant than that observed in H98 (Fig. 4A and B). These results are consistent with the idea that the *H. volcanii* prepilin peptidase processes its flagellins. Moreover, preflagellins appear not to be incorporated into surface filaments, resulting in a minor subset of unprocessed membrane-associated FlgA2.

Using FlaFind, a program that identifies genes that are likely to encode archaeal type IV pilin-like proteins, *in silico* analyses of the *H. volcanii* genome have identified genes that encode proteins resembling type IV pilins (25, 47, 48). To determine whether PibD could process nonflagellin subunits, we overexpressed two of the *H. volcanii* FlaFind positives (Hvo\_A0632 or Hvo\_02451) fused to a C-terminal His tag in *H. volcanii* H98 as well as MT3. Consistent with the facts that these putative pilin-like proteins contain predicted class III signal peptides at their N termini and that PibD has a broad substrate specificity, the putative pilins expressed in H98 were processed while those expressed in MT3 appeared to be unprocessed (Fig. 4B). Conversely, the *H. volcanii* S-layer glycoprotein, which has been shown to have a class I Sec signal peptide (51), migrated at the same position in an SDS-polyacrylamide gel, regardless of whether it was from an H98 or MT3 cell extract. This result suggests that, as expected, the lack of the prepilin peptidase does not have an indirect effect on class I signal peptide cleavage of the glycoprotein precursor (Fig. 4B).

**PibD is required for swimming motility and surface adhesion.** Similar to the  $\Delta flgA1 \Delta flgA2$  strains (MT1 and MT2), *H. volcanii*  $\Delta pibD$  strains (MT3 and MT4) grow as well as the parental strains (H98 and H53, respectively) in complex and defined media (Table 3). These deletion strains are also competitively equivalent to the wild-type strain under both standard and modified growth conditions (Table 3). As with the  $\Delta flgA1 \Delta flgA2$  strain,  $Ca^{2+}$ -induced aggregation as well as conjugation of the H98  $\Delta pibD$  and H53  $\Delta pibD$  (MT3 and MT4) strains seems unaffected by the *pibD* deletion (Table 3), and consistent with a lack of flagella, the  $\Delta pibD$  strains are not motile in CX-0.3% agar medium (Fig. 2B). However, unlike

the  $\Delta flgA1 \Delta flgA2$  strain, the adherence of the  $\Delta pibD$  strain to the glass coverslip is severely defective as only a negligible number of cells are observed at the air-liquid interface (Fig. 6). Since the MT8 strain containing a plasmid that expresses PibD under the control of the inducible *trp* promoter is motile and can adhere to glass when grown in the presence of tryptophan, the phenotypes of the  $\Delta pibD$  strain are thus strongly correlated to the lack of PibD (Fig. 2B and 6). Hence, while surface structures other than type IV pili appear to be involved in aggregation and conjugation, this strongly suggests that type IV pilus-like structures are involved in *H. volcanii* surface adhesion.

## DISCUSSION

Microorganisms have developed many strategies to escape stress conditions. Motility allows the cells to move toward nutrients and away from toxins; aggregation on surfaces (biofilms) or in liquid medium protects cells from harmful extracellular conditions and promotes DNA exchange. Neither swimming motility nor surface adhesion had been reported for *H. volcanii* prior to this study. In addition to determining medium conditions that support these cellular processes in this widely used archaeal model organism, we have also identified a number of genes that encode proteins which play critical roles in these processes.

While the conditions used previously to scrutinize *H. volcanii* motility produced a negative result, we have now defined two distinct media in which this haloarchaeon is motile. We have also shown that an in-frame deletion of the *flgA1* and *flgA2* flagellin genes renders cells nonmotile and that complementing this deletion mutant with the same genes *in trans* from a plasmid rescues the motility phenotype. Similarly, consistent with the fact that we have demonstrated that the *H. volcanii* prepilin peptidase homolog processes the FlgA2 flagellin, a deletion mutant lacking this peptidase is also

nonmotile, clearly demonstrating that *H. volcanii* motility is dependent on its flagella.

Bacterial and archaeal flagella are also involved in biofilm formation, perhaps providing the motive force necessary to overcome the repulsive forces that exist between a bacterium and a typical abiotic surface (41). Modifying the ALI assay to conform to the high-salt concentrations required by *H. volcanii*, we have shown that *H. volcanii* cells bind to a glass coverslip at the air-liquid interface and that under low-oxygen conditions adhesion is inhibited. It is intriguing that although the *H. volcanii* mutant is unable to swim, the mutant bacteria adhere to a glass surface just as effectively as the wild type. Perhaps haloarchaea, distinct from nonhalophilic prokaryotes, do not require flagella to adhere to surfaces because the high salt concentration in the medium masks the repulsive forces between the charged surfaces.

Interestingly, *pibD* deletion mutants do not bind to a glass surface, and complementing this strain in *trans* with *pibD* expression from a plasmid restores its ability to bind to glass. This result clearly demonstrates that the observed adhesion of *H. volcanii* is not due to nonspecific surface attachment. Moreover, by showing that a *pibD* deletion mutant does not bind to glass coverslips and that PibD is responsible for processing *H. volcanii* pilin-like proteins (Hvo\_2451 and Hvo\_A0632), we have demonstrated that the observed flagellum-independent surface adhesion is very likely to involve type IV pilus-like structures. Interestingly, while our results for the *pibD* deletion mutant are consistent with results produced by studies of prepilin peptidase homologs in bacteria as well as with results obtained for *S. solfataricus* (21, 26–28, 37), to date, we have not observed adherence to plastic surfaces, nor have we seen UV-induced autoaggregation. Our previous comprehensive *in silico* analysis of the *H. volcanii* genome identified five operons, each of which contains at least two genes encoding type IV pilin-like proteins (25). Under the conditions that we have tested for surface adhesion, microarray data gathered by Charles Daniels' group indicates that two of these operons are either not expressed or are expressed at very low levels (C. Daniels, personal communication). Finding the conditions under which these operons are expressed will be crucial to a complete understanding of the roles type IV pilus-like structures play in *H. volcanii*.

While we have not yet identified conditions under which UV light induces autoaggregation, we have observed Ca<sup>2+</sup>-dependent aggregation. Interestingly, effective *H. volcanii* Ca<sup>2+</sup>-induced aggregation requires neither flagella nor the prepilin peptidase. Moreover, the conjugation frequencies between two distinct auxotrophic *H. volcanii* *pibD* deletion strains, whether determined on filter plates or in liquid medium, are comparable to the frequencies observed for the wild type. Consistent with these results, we have observed protein complexes on the cell surfaces of *H. volcanii*  $\Delta$ *flgA1*  $\Delta$ *flgA2* and  $\Delta$ *pibD* strains (data not shown) using electron microscopy. In fact, we have not yet discerned any differences between the surfaces of these deletion strains and the wild type. Clearly, the knockout and complementation studies carried out reveal that motility is flagellum dependent. Perhaps the structures encoded by the deleted genes are sheered off during the experimental procedures prior to electron microscopy observations, or, alternatively, the missing structures may be very similar in appearance

to some of the structures that remain. The presence of a diverse set of type IV pilus-like structures and other distinct types of filaments on the surface of *H. volcanii* reflects the critical roles that cell surface structures play in cellular processes in all domains of life. Comparing and contrasting the roles and relationships of these various structures, both within and between domains, will have a significant impact on our understanding of cell biology and all that this entails, from pathogenesis to survival in extreme environments.

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