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Different Minimal Signal Peptide Lengths Recognized by the Archaeal Prepilin-Like Peptidases FlaK and PibD▽

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In *Archaea*, the preflagellin peptidase (a type IV prepilin-like peptidase designated FlaK in *Methanococcus voltae* and *Methanococcus maripaludis*) is the enzyme that cleaves the N-terminal signal peptide from preflagellins. In methanogens and several other archaeal species, the typical flagellin signal peptide length is 11 to 12 amino acids, while in other archaea preflagellins possess extremely short signal peptides. A systematic approach to address the signal peptide length requirement for preflagellin processing is presented in this study. *M. voltae* preflagellin FlaB2 proteins with signal peptides 3 to 12 amino acids in length were generated and used as a substrate in an in vitro assay utilizing *M. voltae* membranes as an enzyme source. Processing by FlaK was observed in FlaB2 proteins containing signal peptides shortened to 5 amino acids; signal peptides 4 or 3 amino acids in length were unprocessed. In the case of *Sulfolobus solfataricus*, where the preflagellin peptidase PibD has broader substrate specificity, some predicted substrates have predicted signal peptides as short as 3 amino acids. Interestingly, the shorter signal peptides of the various mutant FlaB2 proteins not processed by FlaK were processed by PibD, suggesting that some archaeal preflagellin peptidases are likely adapted toward cleaving shorter signal peptides. The functional complementation of signal peptidase activity by FlaK and PibD in an *M. maripaludis* Δ*flaK* mutant indicated that processing of preflagellins was detected by complementation with either FlaK or PibD, yet only FlaK-complemented cells were flagellated. This suggested that a block in an assembly step subsequent to signal peptide removal occurred in the PibD complementation.

The bacterial type IV prepilin peptidase (TFPP) is a well-characterized enzyme belonging to a family of novel aspartic acid proteases (20). It is responsible for the cleavage of N-terminal signal peptides from prepilins and pseudopilins, prior to their incorporation into the type IV pilus structure (22, 30, 31). The prepilin peptidase is also responsible for the processing of prepilin-like proteins needed for type II secretion (22). In *Archaea*, the existence of bacterial TFPP-like enzymes has also been reported, and they have been most extensively studied in relation to the assembly of the archaeal flagellum. In the euryarchaeotes *Methanococcus maripaludis* and *Methanococcus voltae*, the preflagellin peptidase FlaK was demonstrated to be responsible for cleaving the N-terminal signal peptide from the preflagellin prior to its incorporation into the growing flagellar filament, a step essential to flagellar assembly (6, 7, 26). In *Sulfolobus solfataricus*, an acidophilic crenarchaeote, the equivalent enzyme, PibD, was also shown to process preflagellins (4). Site-directed mutagenesis of FlaK and PibD demonstrated that both aspartic acid residues that aligned with aspartic acid residues essential for bacterial TFPP activity were also essential in the archaeal enzymes (6, 32), indicating that the two archaeal peptidases belong with the bacterial TFPPs in this novel family of aspartic acid proteases (20). More recently, an additional archaeal TFPP was found to be required for cleavage of the prepilin substrates (33) that are assembled into the unique pili of *M. maripaludis* (37).

The substrate specificity of the archaeal preflagellin peptidase remains an open question. Like prepilin peptidases, FlaK in *M. voltae* has stringent requirements for the amino acids surrounding the cleavage site of the substrate, especially the −1 glycine, −2 and −3 lysines, and the +3 glycine (numbers given relative to the cleavage site) (35); the last position was conserved in all archaeal flagellins (25). Upon N-terminal sequence alignment of all available archaeal flagellin amino acid sequences at the predicted cleavage site, it was found that most archaeal preflagellin signal peptides are quite conserved in length, with the typical flagellin signal peptide being 11 to 12 amino acids in length (Table 1). It is speculated that while a certain amount of flexibility might exist, some optimum and minimum length probably exists that is crucial for the juxtaposition of the signal peptide and signal peptidase with respect to each other and the membrane (18). A recent study examining possible type IV pilin-like substrates in archaea using the FlaFind program indicated that such substrates may be more widespread than initially thought (33). Since in *Methanococcus* the pilins are processed by a second TFPP (EppA) (33), it is very possible that the preflagellins might be the only substrates of FlaK in these archaea.

Studies on PibD in *S. solfataricus*, however, present interesting disparities. A recent genomic survey revealed a surprisingly large group of proteins possessing type IV pilin-like signal peptides in *Sulfolobus* compared to other archaea (2, 33). Be-

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TABLE 1. N-terminal amino acid alignment of selected archaeal flagellin sequences[a]

Organism	Flagellin	N-terminal sequence
Archaeoglobus fulgidus	FlaB1	**MGMRFLKNEKG**FTGLEAAIVLIAFVTVAAVFSYVLL
Aeropyrum pernix	FlaB1	**MRRRRG**IVGIEAAIVLIAFVIVAAALAFVAL
Haloarcula marismortui	FlaA	**MFEKIANENERG**QVGIGTLIVFIAMVLVAAIAAGVLI
Halobacterium salinarum	FlgA1	**MFEFITDEDERG**QVGIGTLIVFIAMVLVAAIAAGVLI
Methanocaldococcus jannaschii	FlaB1	**MKVFEFLKGKRG**AMGIGTLIIFIAMVLVAAVAAAVLI
Methanococcoides burtonii	Fla	**MKANKHLMMNNDRA**QAGIGTLIIFIAMVLVAAVAAAVLI
Methanococcus aeolicus	Fla	**MNLEHFSFLKNKKG**AMGIGTLIIFIAMVLVAAVAASVLI
Methanococcus maripaludis	FlaB1	**MKIKEFLKTKKG**ASGIGTLIVFIAMVLVAAVAASVLI
Methanococcus vannielii	FlaB1	**MSVKNFMNNKKG**DSGIGTLIVFIAMVLVAAVAASVLI
Methanococcus voltae	FlaB2	**MKIKEFMSNKKG**ASGIGTLIVFIAMVLVAAVAASVLI
Methanothermococcus thermolithotrophicus	FlaB1	**MKIAQFIKDKKG**ASGIGTLIVFIAMVLVAAVAASVLI
Methanogenium marisnigri	Fla	**MKRQFNDNA**FTGLEAAIVLIAFIVVAAVFSYVVL
Methanospirillum hungatei	Fla	**MNNEDG**FSGLEAMIVLIAFVVVAAVFAYATL
Natrialba magadii	FlaB1	**MFEQNDDRDRG**QVGIGTLIVFIAMVLVAAIAAGVLI
Natronomonas pharaonis	Flg1	**MFETLTETKERG**QVGIGTLIVFIALVLVAAIAAGVLI
Pyrococcus abyssi	FlaB1	**MRRG**AIGIGTLIVFIAMVLVAAVAAGVLI
Pyrococcus furiosus	Fla	**MKKG**AIGIGTLIVFIAMVLVAAVAAGVLI
Pyrococcus horikoshii	FlaB1	**MRRG**AIGIGTLIVFIAMVLVAAVAAAVLI
Sulfolobus solfataricus	Fla	**MNSKKMLKEYNKKVKRKG**LAGLDTAIILIAFIITASVLAYVAI
Sulfolobus tokodaii	Fla	**MGAKNAIKKYNKIVKRKG**LAGLDTAIILIAFIITASVLAYVAI
Thermococcus kodakarensis	FlaB1	**MKTRTRKG**AVGIGTLIVFIAMVLVAAVAAAVLI
Thermoplasma acidophilum	Fla	**MRKVFSLKADNKA**ETGIGTLIVFIAMVLVAAVAATVLI
Thermoplasma volcanium	Fla	**MYIVKKMPILKLLNSIKRIFKTDDSKA**ESGIGVLIVFIAMILVAAVAASVLI

[a] In all organisms listed, except *Sulfolobus*, there are multiple flagellins but only a single example is shown. The signal peptide is shown in boldface type. In some cases, analyses of the amino acid sequences of the signal peptides with unusual lengths revealed in-frame methionines or alternative start sites (underlined) that, if they represent the true translation start site, would result in signal peptides of more typical lengths. For *S. solfataricus*, Albers et al. (4) used the internal start site to give a signal peptide of 13 amino acids and demonstrated signal peptide processing.

sides the preflagellins, other substrates for PibD include pilins and proteins involved in sugar binding. Deletions of *pibD* appear to be nonviable (1), unlike the case for *flaK*, reinforcing the role of *pibD* in processes other than flagellum and pilus formation. Site-directed mutagenesis on the glucose-binding protein precursor (GlcS) signal peptide revealed that a wide variety of substitutions around the cleavage site still permitted processing. The allowed substitutions were consistent with the signal peptide sequences of a list of proposed PibD substrates, some of which have predicted signal peptides as short as 3 amino acids (4). Based on the observation that homologues of *S. solfataricus* sugar-binding proteins that contain type IV prepilin-like sequences were absent in the genome of another species of *Sulfolobus*, *Sulfolobus tokodaii*, it was speculated that *S. solfataricus* PibD may have undergone a specialization allowing for a broader substrate specificity (4). However, whether the extremely short signal peptides would be functional and recognizable as preflagellin peptidase substrates remains to be biochemically demonstrated.

Although the typical flagellin signal peptide is 11 to 12 amino acids in length, a small number of archaeal preflagellins contain signal peptides of unusual lengths. Some are annotated to be unusually long (e.g., MJ0893 of *Methanocaldococcus jannaschii* and Ta1407 of *Thermoplasma acidophilum*) (Table 1). These sequences, however, contain in-frame alternative translational start sites that, if they correspond to true translation start sites, would result in signal peptides more typical in length. On the other hand, organisms with preflagellins predicted to possess unusually short signal peptides of 4 to 6 amino acids include *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Pyrococcus horikoshii*, and *Aeropyrum pernix* (Table 1). These unusual signal peptides are deduced exclusively from gene sequences. Biochemical or genetic data to explain these peculiarities are still lacking. Assuming that the annotations of these genes are accurate, this would suggest that certain archaeal TFPP-like enzymes possess the capacity to process these much shorter signal peptides.

In this study, for the first time, a systematic evaluation of critical signal peptide length for recognition and cleavage by two very different archaeal TFPP-like signal peptidases, *M. voltae* FlaK and *S. solfataricus* PibD, is reported.

MATERIALS AND METHODS

Microbial strains and growth conditions. *M. maripaludis* LL, *M. maripaludis* Mm900 (Δ*hpt*) (23) and *M. voltae* PS were routinely grown in Balch medium III at 30°C or 37°C under an atmosphere of CO_2-H_2 (20:80) as previously described (19). For the in-frame deletion mutagenesis experiments in *M. maripaludis* Mm900, cells were grown in McCas medium supplemented with 8-azahypoxanthine (240 μg/ml) or neomycin (1 mg/ml) as required in the procedure as described previously (23). In complementation experiments, the plasmids were maintained in *M. maripaludis* by puromycin (2.5 μg/ml) selection. For induction of protein expression under the control of the *nif* promoter in *M. maripaludis*, cells were grown in nitrogen-free medium (10) supplemented with 100 mM NH_4Cl (promoter-off conditions) or 100 mM alanine (promoter-on conditions) (21). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) when necessary. *E. coli* DH5α (Novagen) was used for all cloning steps. *E. coli* BL21(DE3)/pLysS (Novagen) was used as the protein expression strain. The strains, plasmids, and primers used in this study are listed in Table 2.

Cloning of the preflagellin gene. To generate variations of the *M. voltae* preflagellin FlaB2 protein with different signal peptide lengths for use in the in vitro peptidase assay, a series of PCR forward primers was designed with in-frame alternative start sites (Table 2). The same reverse primer was used throughout. The forward and reverse primers were designed with NdeI and XhoI restriction sites incorporated, respectively. PCR was performed using *Pwo* DNA polymerase under the following conditions: 95°C for 5 min; 29 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min; and a final cycle with an extension time of 5 min at 72°C. The amplified product was purified using a PCR purification

TABLE 2. Strains, primers, and plasmids used in this study

Strain/construct or purpose	Source or reference	Primer		Corresponding plasmid
		Name	Sequence (5′–3′)[a]	
M. voltae P2	W. B. Whitman			
M. maripaludis LL	J. Leigh			
M. maripaludis Mm900	J. Leigh			
E. coli BL21(DE3)/pLysS	Novagen			
E. coli DH5α(pET23a+/Mvo flaB2 LP12)	35			pKJ284
E. coli DH5α(pET23a+/Mvo flaB2 LP10)	This study	P_{for}	GGAATTCCATATGAAAGAATTCATGAGTAACAAA AAAGG (NdeI)	pKJ483
E. coli DH5α(pET23a+/Mvo flaB2 LP9)	This study	P_{for}	GGAATTCCATATGGAATTCATGAGTAACAAAA AAGG (NdeI)	pKJ465
E. coli DH5α(pET23a+/Mvo flaB2 LP8)	This study	P_{for}	GGAATTCCATATGTTCATGAGTAACAAAAAAGG (NdeI)	pKJ474
E. coli DH5α(pET23a+/Mvo flaB2 LP7)	This study	P_{for}	GGAATTCCATATGATGAGTAACAAAAAAGGTGC (NdeI)	pKJ473
E. coli DH5α(pET23a+/Mvo flaB2 LP6)	35			pKJ287
E. coli DH5α(pET23a+/Mvo flaB2 LP5)	This study	P_{for}	GGAATTCCATATGAACAAAAAAGGTGCTTCAGGA ATTGG (NdeI)	pKJ509
E. coli DH5α(pET23a+/Mvo flaB2 LP4)	This study	P_{for}	GGAATTCCATATGAAAAAAGGTGCTTCAGGAA TTGG (NdeI)	pKJ519
E. coli DH5α(pET23a+/Mvo flaB2 LP3)	This study	P_{for}	GGAATTCCATATGAAAGGTGCTTCAGGAATTGGT ACC (NdeI)	pKJ511
Common reverse primer for flaB2 constructs	This study	P_{rev}	CCGCTCGAGTATTGTAATTGAACTACT (XhoI)	
E. coli DE3(pACYC-RIL)/ His_6-Sso PibD	Z. Szabo			pKJ595
Deletion of *M. maripaludis flaK*	This study	P_{for} 2kb_up	CGGATCCCTCGTGCAGTATCAGCAATG (BamHI)	pKJ574
	This study	P_{rev} 2kb_up	TGGCGCGCCTGTATTCTATCAAACTATCG (AscI)	
	This study	P_{for} 2kb_down	TGGCGCGCCTTCTAAAAATCGATTTCAATCC (AscI)	
	This study	P_{rev} 2kb_down	CGGATCCTCGTTTAGACCTCCAAGAGA (BamHI)	
PCR screening of *flaK* deletion mutant	This study	P_{for}	AATATCTGGCGGATACAGG	
	This study	P_{rev}	TTCAAAGCCAATAGATACTGC	
M. maripaludis flaK complementation (*nif*)	This study	P_{for}	CCAATGCATGATAGAATACATCATTGGAGTA ATCG (NsiI)	pKJ619
	This study	P_{rev}	CGACGCGTTCAGTGATGGTGGTGATGATGACGAC CCTCAATGGATCCGAATGGAATTAAAAGAT CAAG (MluI)	
S. solfataricus pibD complementation (*nif*)	This study	P_{for}	CCAATGCATGGTCGTTATATATTATATCCAAAT (NsiI)	pKJ620
	This study	P_{rev}	CGACGCGTTCAGTGATGGTGGTGATGATGACGAC CCTCAATGG (MluI)	

[a] Restriction sites are underlined, and restriction enzymes are given in parentheses.

column (Qiagen, Chatsworth, CA) and cloned into the MCS of the pET23a(+) vector via the NdeI and XhoI restriction sites. All the constructs were checked by DNA sequencing to ensure that no errors occurred during the PCRs.

In-frame deletion of preflagellin peptidase *flaK* in *M. maripaludis*. An *M. maripaludis flaK* in-frame deletion strain was created according to published methodology (23). Briefly, primers were designed to PCR amplify separately the upstream and downstream flanking sequences of *flaK* using *M. maripaludis* genomic DNA as a template. Primers Prev2kb_up and Pfor2kb_down had AscI sites incorporated so that the two PCR products could be digested and ligated together. Another round of PCR with primers Pfor2kb_up and Prev2kb_down allowed for the amplification of a 2-kb fragment containing an in-frame deletion within the *flaK* gene (19 bp left of the 693 bp open reading frame). This product was cloned into pCRPrtNeo via the flanking BamHI sites, generating pKJ593. Sequencing was performed to confirm the cloned piece, making sure that the subsequent deletion would be in frame. To generate a strain carrying a deletion in *flaK*, *M. maripaludis* Mm900 was transformed with pKJ593 using the methodology of Tumbula et al. (36), with all the steps performed anaerobically. Briefly, 5 ml of freshly grown *M. maripaludis* Mm900 was washed and resuspended in transformation buffer (50 mM Tris, 0.35 M sucrose, 0.38 M NaCl, 1 mM $MgCl_2$, 0.00001% rezasurin, pH 7.5). The resuspended culture was mixed with 5 μg of DNA in the presence of polyethylene glycol (18%, wt/vol). The transformation mix was incubated for 1 h, inoculated into 10 ml McCas medium, and incubated overnight. Subsequently, the culture was subcultured in medium with neomycin (for selection for vector integration) and incubated at 37°C overnight. This culture was then used to inoculate McCas medium without neomycin selection to allow for a second recombination event to remove the vector, leaving cells with either the wild-type version of the gene or the in-frame deletion version.

The culture was plated onto McCas agar containing 8-azahypoxanthine, which would be lethal to any cells that retained the pKJ593 vector-borne *hpt* gene. The plates were incubated at 37°C in an anaerobic canister (CO_2-H_2 pressurized, with 3 ml of 25% sodium sulfide). Single colonies were picked after 5 days and grown in Balch medium III, followed by screening by PCR and Southern blot analysis to identify deletion mutants.

PCR screening of deletion mutants. Internal PCR primers were designed to provide a PCR screen of the transformants for gene deletion. Overnight cultures of transformants picked from single colonies on 8-azahypoxanthine-containing plates were washed twice with 2% NaCl and used directly as PCR templates. The PCR was performed with *Taq* DNA polymerase with the following cycles: 95°C for 5 min; 29 cycles of 94°C for 45 s, 45°C for 45 s, and 72°C for 1 min; and a final cycle with an extension time of 10 min at 72°C. The PCR products were electrophoresed on 0.8% agarose gels for analysis, alongside a 100-bp ladder marker (New England Biolabs, MA).

Southern blot analysis of the deletion mutants. PCR primers were used to amplify, from *M. maripaludis* genomic DNA, a ~1 kb fragment across the deletion region. The amplified product was subsequently used to generate a

digoxigenin-labeled probe using a DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Southern blotting was carried out as previously described using a hybridization temperature of 50°C (34).

Cloning of *M. maripaludis flaK* and *S. solfataricus pibD* for complementation into an *M. maripaludis* Δ*flaK* strain. The expression vector pHW40 (obtained from J. Leigh) was used for cloning *flaK* and *pibD* for complementation in *M. maripaludis* Δ*flaK*. The vector has the gene of interest cloned under the inducible *nif* promoter. The genes of interest were amplified by PCR using forward and reverse primers with NsiI and MluI restriction sites incorporated, respectively. PCR was performed using *Pwo* DNA polymerase under the following conditions: 95°C for 5 min; 29 cycles of 94°C for 45s, 50°C for 45s, and 72°C for 1 min; and a final cycle with an extension time of 5 min at 72°C. The PCR products contained in-frame C-terminal His tags from the primers so that, when overexpressed, the gene products could be tracked by immunoblotting. The amplified product was purified using a PCR purification column (Qiagen, Chatsworth, CA) and cloned into pHW40 via the NsiI and MluI restriction sites. Transformation of *M. maripaludis* Δ*flaK* was performed in an anaerobic chamber as previously described; this culture was plated onto Balch III medium agar containing puromycin and incubated at 37°C in an anaerobic canister (CO_2-H_2 pressurized, with 3 ml of 25% sodium sulfide). Single isolated transformant colonies were picked after 5 days. These cells were grown in N-free medium supplemented with puromycin and either NH_4 or alanine. Cells were grown at both 30 and 37°C and then examined by light microscopy for motility, by electron microscopy for appearance of flagella, and by Western blotting for evidence of flagellin processing.

Overexpression of the *M. voltae* preflagellin and *S. solfataricus* signal peptidase for the in vitro assays. Membranes containing overexpressed *M. voltae* preflagellin were obtained from *E. coli* BL21(DE3)/pLysS cells harboring pKJ91 (9, 12). Protein expression was induced in log phase cells with the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to 0.4 mM. After a 90-min induction, cells were harvested by centrifugation (5,000 × *g* for 10 min), and the pellet was frozen overnight. The pellet was resuspended in 20 mM Tris-HCl buffer (pH 8.0), and the viscous solution was sonicated four times for 30 s each time on ice to aid cell lysis. Unbroken cells and potential inclusion bodies were removed by centrifugation at 5,000 × *g* for 5 min. A crude membrane fraction containing the overexpressed preflagellin was then obtained by centrifugation of the supernatant at 20,000 × *g* for 30 min. The pellet was resuspended in 500 μl of distilled H_2O (dH_2O). This material was used in subsequent peptidase assays as a substrate source.

E. coli pACYC-RIL harboring *S. solfataricus pibD* was obtained from Z. Szabo (Haren, The Netherlands). Protein expression was induced in log phase cells with the addition of IPTG to 0.4 mM. Upon addition of IPTG, cells were shifted to 30°C and grown for 18 h (4). Cells were harvested by centrifugation (5,000 × *g* for 10 min), and the pellet was kept frozen overnight. To obtain a crude membrane fraction, the cells were subjected to lysis by lysozyme (1 mg/ml final concentration) with the addition of 1% Triton X-100 for 15 min at 30°C. A crude membrane fraction was prepared as above, except that the final resuspension was in 200 μl of assay buffer (25 mM morpholinethanesulfonic acid, 0.5% Triton X-100, 150 mM KCl, 1 mM EDTA, pH 6.5) (4). This material was used in subsequent peptidase assays as enzyme source.

Isolation of *M. voltae* and *M. maripaludis* membranes as an enzyme source for the in vitro assays. Six milliliters of late-exponential-phase *M. voltae* or *M. maripaludis* cells were harvested by aerobic centrifugation at 16,000 × *g* for 5 min. The pellets were resuspended in 100 μl of medium and diluted with sterile dH_2O to a final volume of 1.5 ml, resulting in lysis of the osmotically fragile cells. The lysate was centrifuged at 16,000 × *g* for 10 min to pellet the membranes, which were resuspended in 100 μl of dH_2O for use in the peptidase assay.

Preflagellin peptidase assays. The assay for *M. voltae* preflagellin peptidase activity was performed as previously described (9, 12). The substrate for the assay was the *E. coli* membrane preparation containing the various forms of overexpressed preflagellin, isolated as described above. The enzyme source was an *M. voltae* membrane preparation. The reaction was started by the addition of *M. voltae* membranes and kept at 37°C in a water bath. Samples (10-μl aliquots) were taken from the reaction mixture at various time points, mixed into 2× electrophoresis sample buffer (0.0625 M Tris-HCl [pH 6.8], 1% [wt/vol] sodium dodecyl sulfate, 10% glycerol, 2% 2-mercaptoethanol, 0.001% bromophenol blue), and immediately boiled for 5 min prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis using anti-FlaB2 antibodies.

The peptidase assays using the cloned *S. solfataricus* signal peptidase were performed in a similar manner under conditions reported previously for the PibD peptidase (25 mM morpholinethanesulfonic acid, pH 6.5, 150 mM KCl, 0.5% Triton X-100, and 1 mM EDTA at 55°C) (4). The enzyme source was an *E. coli* membrane preparation containing overexpressed *S. solfataricus* PibD, with an *E. coli* membrane preparation containing the various forms of overexpressed preflagellin as a substrate.

The peptidase assay of the complementation strains of *M. maripaludis*, MmΔ*flaK*(pHW40::*flaK*) and MmΔ*flaK*(pHW40::*pibD*), were performed as previously described for the *M. voltae* preflagellin peptidase assay. The enzyme source was an *M. maripaludis* membrane preparation from cells expressing either *M. maripaludis* FlaK or *S. solfataricus* PibD.

Electron microscopy. To prepare samples for electron microscopy, *M. maripaludis* cells were grown overnight in 10 ml of appropriate medium. The cells were briefly washed and resuspended in phosphate-buffered saline. Samples were negatively stained with 2% phosphotungstic acid and supported on carbon-Formvar-coated copper grids. They were examined in a Hitachi 7000 electron microscope operating at 75 kV.

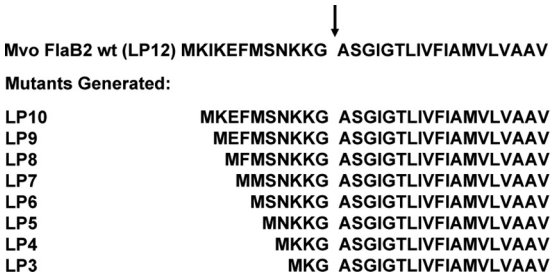

FIG. 1. N-terminal amino acid sequence of *M. voltae* FlaB2 and mutants generated for this study. The vertical arrow indicates the site at which cleavage of the signal peptide from the preflagellin by FlaK occurs.

RESULTS

***M. voltae* FlaK processes preflagellins with a minimum signal peptide length of 5 amino acids.** To systematically address the relationship between signal peptide length and proper processing by the preflagellin peptidase FlaK, *M. voltae* mutant FlaB2 proteins with truncated signal peptides were generated and used as a substrate in an in vitro preflagellin peptidase assay utilizing *M. voltae* membranes as an enzyme source. In all the mutant proteins, the conserved amino acids surrounding the cleavage site were unaltered (Fig. 1). All of the mutant versions of FlaB2 were expressed to similar levels, and all appeared stable in *E. coli*. Significant processing was observed in all FlaB2 proteins containing signal peptides shortened from 12 to 5 amino acids with the exception of LP6, whereas flagellins with signal peptides at or below 4 amino acids in length were not processed (Fig. 2). The inability of FlaK to process preflagellins with a signal peptide shortened to 6 amino acids was previously reported (35). Thus, besides the amino acids surrounding the cleavage site, it appears that signal peptide length is also crucial for proper processing by the *M. voltae* enzyme.

***S. solfataricus* PibD has a less stringent signal peptide length requirement for processing.** Since the flagellin processing enzyme of *S. solfataricus*, PibD, is more flexible in its substrate requirements than FlaK and may have natural substrates with very short signal peptides, the ability of this signal peptidase to cleave the collection of methanogen FlaB2 substrates was examined. A crude *E. coli* membrane preparation, containing a His-tagged version of PibD, was used as an enzyme source in an in vitro assay adapted for the *S. solfataricus* enzyme. Interestingly, besides cleaving the longer signal peptides that *M.*

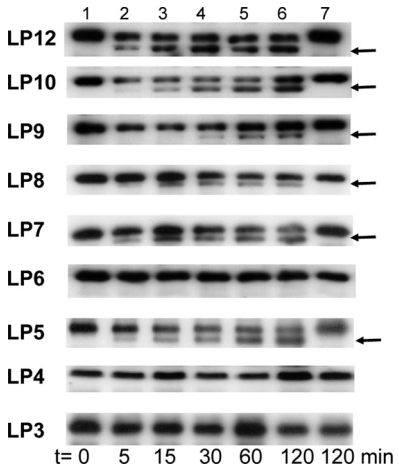

FIG. 2. In vitro analysis of mutant preflagellin processing by *M. voltae* membranes. The preflagellin peptidase assay was performed on *M. voltae* preflagellin with signal peptides of various lengths at 37°C. From each reaction mixture, samples were taken at various time points, as indicated below the panel. Controls with sterile dH_2O substituting for *M. voltae* membranes were also performed (lane 7). The reaction was quenched through the addition of 2× electrophoresis sample buffer, followed by boiling for 5 min. Peptidase activity against the preflagellin protein was detected via immunoblotting with chicken polyclonal anti-FlaB2 antibodies (1/2,500) as a primary antibody. Arrows indicate the processed flagellins. *t*, time.

voltae processed, PibD also successfully recognized and cleaved the shorter signal peptides that were not processed by *M. voltae* FlaK (Fig. 3). This included the preflagellin with the 6-amino-acid signal peptide, showing that the inability of the

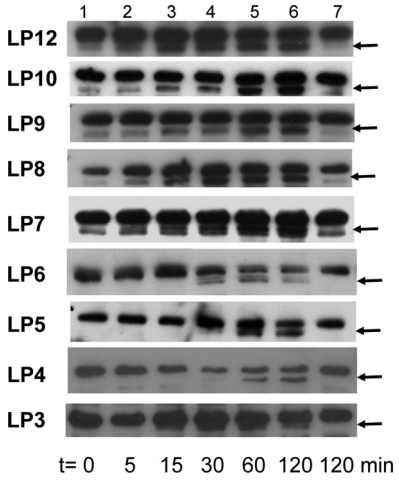

FIG. 3. In vitro analysis of mutant preflagellin processing by *S. solfataricus* PibD. The peptidase assay was adapted for testing *S. solfataricus* PibD activity. The assay was performed at 55°C. *M. voltae* preflagellin with various signal peptide lengths was used as a substrate as indicated. An *E. coli* membrane preparation containing heterologously expressed PibD was used as the enzyme source. From each reaction mixture, samples were taken at various time points, as indicated below the panel. Controls with sterile dH_2O substituting for the enzyme source were also performed (lane 7). In the case of LP3, 17.5% acrylamide gel as opposed to 15% was needed for separation of the processed and unprocessed flagellins. Arrows indicate the processed flagellins. *t*, time.

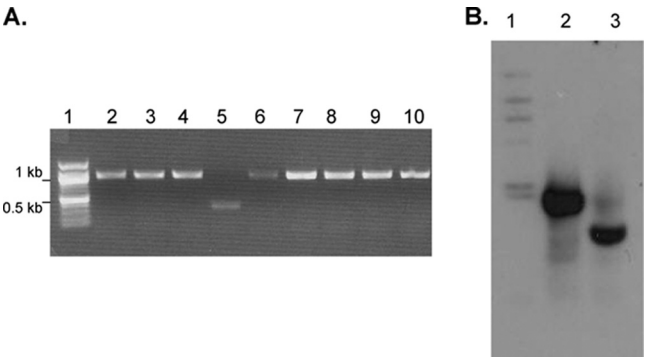

FIG. 4. (A) Identification of a *flaK* deletion mutant by a PCR screen of individual transformants. The PCR primers were designed to amplify across the *flaK* gene region and give a 1,000-bp product if the *flaK* gene is intact and a 300-bp product if the *flaK* gene is deleted. Of the nine transformants tested in this run, only number 5 was a mutant. (B) Confirmation of *flaK* deletion by Southern blotting. Genomic DNA was digested with RsaI and hybridized with a probe specific for the *flaK* surrounding region. Wild-type (lane 2) and mutant DNA (lane 3) yielded bands that matched the predicted sizes of 2.1 kb and 1.4 kb, respectively. Lane 1 is λ-HindIII-digested DNA marker (23, 9, 6.5, 4.3, 2.3, 2, and 0.5 kb; the last marker band is not seen on this gel).

M. voltae membranes containing FlaK to process this substrate was not due to an inherent inability of this substrate to be processed. The assays were performed at 55°C (4). However, the enzyme was also proven functional at 37°C both in this study (with LP12 as a substrate [data not shown]) and previously (4) although the reaction is less efficient at the lower temperature, which is not unexpected since *S. solfataricus* grows optimally near 80°C. The ability of this thermophilic enzyme to function at this much lower temperature prompted us to explore its ability to functionally complement FlaK in vivo. For these experiments, a deletion of *flaK* was created in *M. maripaludis*, a methanococcal species for which genetic manipulations that allow the generation of in-frame deletions and complementation not yet possible in *M. voltae* have been reported (23).

***M. maripaludis* Δ*flaK* was nonmotile and nonflagellated.** An in-frame *flaK* deletion mutant in *M. maripaludis* was generated following transformation with pKJ593. Transformants were screened by PCR, with mutant cells generating the predicted 300-bp product as opposed to the 1,000-bp product for transformants that retained the wild-type version of *flaK* (Fig. 4A). The *flaK* deletion was further confirmed by Southern blot analysis, with the mutants showing the expected 1.4-kb hybridizing RsaI fragment (Fig. 4B). The *flaK* mutant was nonmotile (by light microscopy examination as well as semi-swarm plate analysis) and nonflagellated although the cells retained several pili on the cell surface (Fig. 5A). Immunoblotting confirmed that the flagellins remained in the cytoplasmic membrane in the unprocessed form only (Fig. 6A). Since the deletion created is markerless, it allowed for complementation studies.

Complementation of the *M. maripaludis flaK* mutant by a plasmid-borne copy of *flaK*. *M. maripaludis* Δ*flaK* was transformed with pHW40 carrying the *flaK* gene, generating MmΔ*flaK*(pHW40::*flaK*). The ability of the complementation to restore preflagellin peptidase function was evaluated through immunoblotting using antiflagellin antiserum to detect

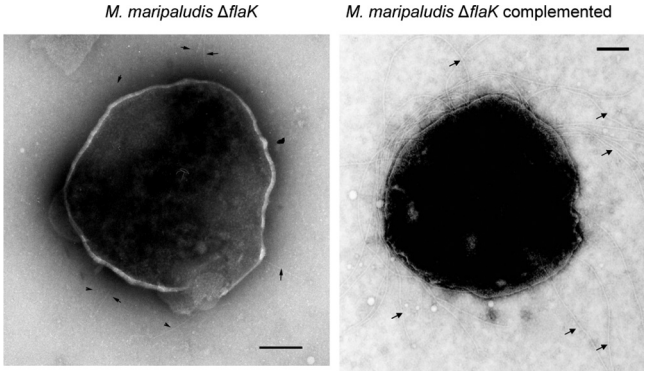

FIG. 5. Electron micrographs of *M. maripaludis* Δ*flaK* (A) and *M. maripaludis* Δ*flaK* (B) cells complemented with plasmid-borne *flaK*. In panel B, cells were grown in N-free medium supplemented with alanine, resulting in expression of *flaK*. Both samples were negatively stained with 2% phosphotungstic acid (pH 7.0). *M. maripaludis* Δ*flaK* cells are nonflagellated, but arrows point out the presence of peritrichously located pili in panel A while complemented cells have flagellation restored in panel B (arrows point to flagella). Bar, 500 (A) and 200 (B) nm.

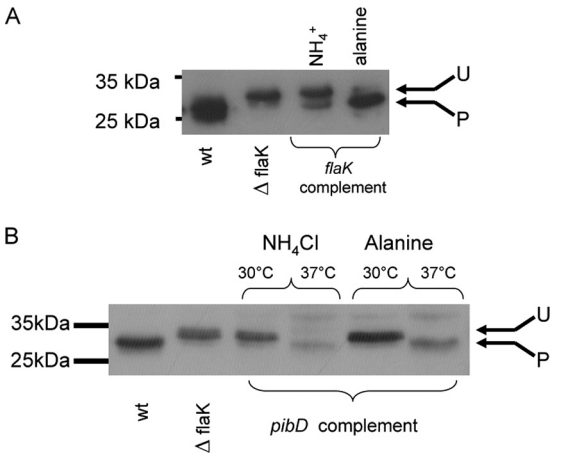

FIG. 6. Immunoblots of *M. maripaludis* Δ*flaK* cells complemented with *flaK* or *pibD*. (A) Immunoblot of *M. maripaludis* crude membrane fractions from wild-type, Δ*flaK*, and Δ*flaK*(pHW40::*flaK*) cells using anti-FlaB2 antibodies (1/2,500) as primary antibodies. Cells were grown in nitrogen-free medium supplemented with alanine (promoter on) or NH_4Cl (promoter off), as indicated. (B) Immunoblot of *M. maripaludis* crude membrane fractions from wild-type, Δ*flaK*, and Δ*flaK*(pHW40::*pibK*) cells using anti-FlaB2 antibodies as primary antibodies. Cells were grown in nitrogen-free medium supplemented with alanine (promoter on) or NH_4Cl (promoter off) and at 30°C or 37°C, as indicated. Wild-type and Δ*flaK* cells were grown at 30°C, but identical blots were obtained when these cells were grown at 37°C. U, unprocessed flagellins; P, processed flagellins.

flagellin processing. The plasmid pHW40 is a self-replicating vector that puts cloned genes under the control of the *nif* promoter and so allows regulated expression of the cloned gene, depending on the nitrogen source in the medium. When expression of *flaK* is on (cells grown in N-free medium supplemented with alanine), processed forms of the flagellins were detected by Western blotting of the crude cell extract representing the majority of flagellins present. Even when expression is off (cells grown in N-free medium supplemented with NH_4^+), a significant band representing processed flagellins was detected although in this case unprocessed flagellins remained the major band in the sample (Fig. 6A). Electron microscopy revealed the presence of flagellar filaments when MmΔ*flaK*(pHW40::*flaK*) was grown in N-free medium supplemented with alanine (Fig. 5B); these cells were motile by light microscopy examination. Crude membrane fractions harvested from MmΔ*flaK*(pHW40::*flaK*) after overnight growth in nitrogen-free medium supplemented with alanine were used as an enzyme source against *M. voltae* preflagellin (LP12) in in vitro assays. Cleavage activity was demonstrated as a conversion of the preflagellins from unprocessed to processed forms over time was detected (data not shown). The stability of the *flaK* deletion in this, as well as of the MmΔ*flaK*(pHW40::*pibD*) construct, was confirmed by PCR (data not shown). Even though activity of FlaK was demonstrated in the in vitro assay, the induced His-tagged FlaK protein itself was undetectable in Western blot analysis using anti-His antibodies (results not shown).

Complementation of the *M. maripaludis flaK* mutant by a plasmid-borne copy of *S. solfataricus pibD*. The in vitro activity of PibD demonstrated it to be active against *M. voltae* preflagellins under conditions of temperature and pH consistent with growth of *M. maripaludis*, suggesting that it might be able to complement the defect in the *flaK* mutant of *M. maripaludis* in vivo. The *pibD* gene was cloned under the *nif* promoter of the expression vector pHW40 to generate MmΔ*flaK*(pHW40::*pibD*). Western blotting using antiflagellin antiserum revealed that when this complementation strain was grown at 30°C in N-free medium supplemented with either NH_4 or alanine, only unprocessed flagellins were detected in the MmΔ*flaK*(pHW40::*pibD*) strain (Fig. 6B). In contrast, when the strain was grown under either growth condition at 37°C, the majority of the flagellins were apparently processed (Fig. 6B). The complemented strain grown under either condition at either temperature, however, was always nonmotile by light microscopy examination and nonflagellated, as revealed by electron microscopy (Fig. 7). In addition, membrane preparations of Mm Δ*flaK*(pHW40::*pibD*) were nonfunctional in in vitro peptidase assays at 30, 37, 42, or 55°C (data not shown). As with FlaK, the His-tagged PibD protein was undetectable in Western blot analysis using anti-His antibodies in uninduced or induced samples at either 30 or 37°C (results not shown).

DISCUSSION

The first reported TFPP-like peptidase in *Archaea* was the preflagellin peptidase FlaK, characterized in the methanogen species *M. maripaludis* and *M. voltae* in association with flagellar biosynthesis (6, 7). FlaK is believed to be a dedicated enzyme for flagellin processing. Recent studies on the homologous enzyme PibD in *S. solfataricus* presented interesting divergences. While FlaK in *M. voltae* has stringent requirements toward amino acids surrounding the cleavage site of the substrate (35), PibD in *S. solfataricus* was shown to be more flexible (32). When the *S. solfataricus* genome was screened for potential substrates for the TFPP-like enzymes using the results of site-directed mutagenesis of known PibD substrates as one of the guides, many potential substrates for PibD were

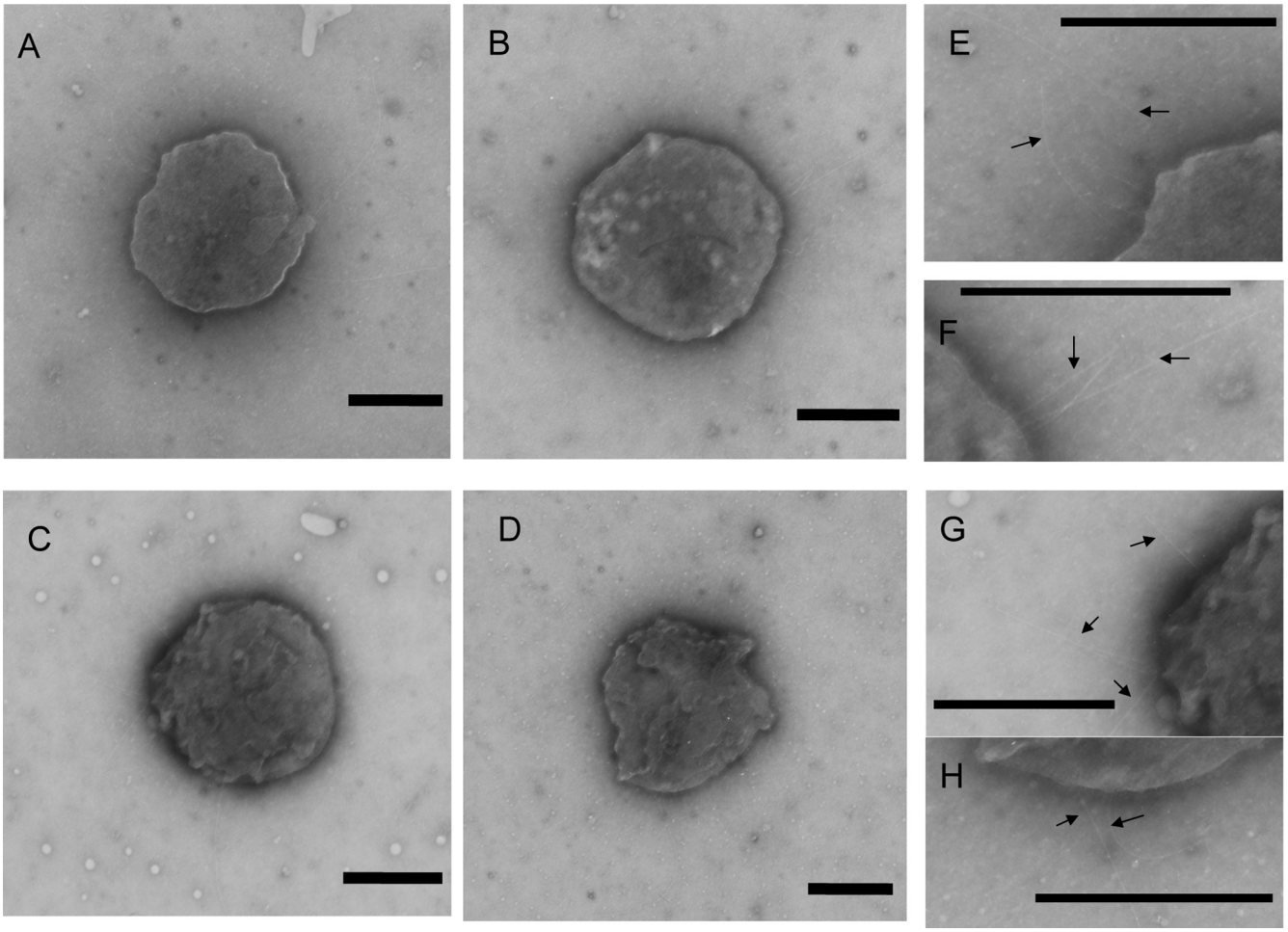

FIG. 7. Electron micrographs of Mm*ΔflaK*(pHW40::*pibD*) cells. Cells were grown in nitrogen-free medium supplemented with alanine or NH_4Cl and at 30°C or 37°C, as indicated. Even at 37°C, where the flagellins appear to be processed (Fig. 6B), the cells are nonflagellated although pili are observed on the cell surface. Growth with NH_4Cl at 37°C (A), with NH_4Cl at 30°C (B), with alanine at 37°C (C), and with alanine at 30°C (D) is shown. Panels E to H are larger magnifications of panels A to D, respectively, which show the presence of pili on the cell surface. Bar, 500 nm.

predicted (32). Our observation that flagellins in some archaeal species have peculiar predicted signal peptide lengths seems to support the view that TFPP-like enzymes may be diverse in *Archaea*. However, this remained an interesting hypothesis without proof since neither these unusual preflagellins from various archaea nor most of the *S. solfataricus* predicted substrates have yet been biochemically validated. The current study was undertaken with the goal of further evaluating the substrate specificities of the two very different archaeal TFPP-like signal peptidases, *M. voltae* FlaK and *S. solfataricus* PibD.

Previous site-directed mutagenesis studies, focused on functional substitutions of amino acids at positions in the immediate vicinity of the substrate cleavage site, suggested that PibD has a less stringent requirement than FlaK (4, 35). The allowed substitutions were consistent with the list of proposed PibD substrates with unusually short signal peptide sequences, thereby suggesting the possibility that PibD has undergone a divergence allowing for recognition and proper processing of substrates with short signal peptides. In assessing the signal peptide length requirements using in vitro assays, it was found that *M. voltae* FlaK has a cutoff of 5 amino acids, below which the substrates remained unprocessed. LP6 was an unusual exception that was never processed despite attempts in this study (Fig. 2) and previously (35). Interestingly, the shorter signal peptides that were not processed by *M. voltae* FlaK were successfully recognized and processed by PibD (Fig. 3). In interpreting these results, it is important to note that, because of the limitations of the in vitro system, there was no true differentiation between interference with the cleavage reaction due to our experimental manipulations versus inaccessibility of the substrate due to misfolding when each assay result was taken alone. However, a comparison of the processing of LP4 and LP3, which are the same substrates that were functional in the PibD assays, strongly suggests that FlaK is subject to a peptide length minimum that is not applicable to PibD. With respect to the nonprocessing of LP6 by FlaK, the ability of PibD to process LP6 clearly indicates that the substrate is capable of being processed. Even though FlaK could not process LP6 for unknown reasons, its ability to process longer and shorter signal peptides would indicate that LP5 is the true cutoff in

terms of signal peptide length. Why the two enzymes have different signal peptide length cutoffs is unknown. Perhaps in the case of FlaK the last five amino acids before the cleavage site are all necessary for effective binding to the peptidase while for PibD perhaps only the last three amino acids are sufficient.

The divergence of the preflagellin peptidase among different archaeal species might be related to the extreme environmental niche and, in turn, physiological challenges faced by the diverse archaeal species. In comparing the proteomes, Albers and Driessen (2) found that approximately 4.2% of the *S. solfataricus* proteome contains putative secretory proteins with N-terminal signal peptides, compared to the predicted 2% for *Methanocaldococcus jannaschii*. There were a relatively large number of proteins with predicted type IV pilin-like signal peptides, many involved in sugar binding and uptake in *S. solfataricus*. *S. solfataricus* contains a lengthy list of sugar-binding proteins, including ones for glucose, galactose, mannose, arabinose, fructose, xylose, cellobiose, and the higher derivatives maltose and maltodextrin as well as trehalose (13). Each of these sugars can serve as the sole carbon source for the growth of *S. solfataricus*. The existence of the many uptake systems is believed to be advantageous for organisms growing in substrate-poor environments, such as hydrothermal vents or hot sulfuric pools, so that they can scavenge all available nutrients (13). The wider than expected utilization of type IV pilin-like signal sequences, as well as the lower enzyme stringency, is most probably a specialization that the PibD enzyme has undergone (4). These additional sugar-binding substrates have been hypothesized to assemble into a pilus-like "bindosome" on the surface of the cell (3, 24).

In view of the limited examples of heterologous complementation in *Archaea*, it was of interest to evaluate whether the unusual PibD could functionally complement a *flaK* deletion in *M. maripaludis*. This is a heterologous complementation across a considerable phylogenetic distance with two organisms with very different physiologies. We confirmed, in an in vitro assay, an earlier report that PibD is functional at 37°C, a temperature much lower than the physiological temperature of *S. solfataricus* but a temperature at which *M. maripaludis* grows well. When *M. maripaludis flaK* was cloned into the complementation vector pHW40 under the *nif* promoter and transformed into *M. maripaludis* Δ*flaK*, the resulting transformant, MmΔ*flaK*(pHW40::*flaK*), had restored flagellation, and crude membrane preparations prepared from this strain were functional in in vitro peptidase assays. These results provided proof for the methodology employed. However, unusual observations were noted when the same complementation was attempted with the *S. solfataricus pibD*. Here, analysis of the state of the flagellins in the complemented cells indicated that processing was occurring at 37°C but not at 30°C. Earlier work had indicated that PibD functions well at 37°C but very poorly at room temperature (4). However, even under conditions where the flagellins were processed, the cells remained nonflagellated, suggesting that, although processed, the flagellins could not feed into the flagellar assembly system. This may be because of an abnormal membrane insertion of PibD due to the very different membrane lipid contents in the cytoplasmic membrane of *S. solfataricus* compared to *M. maripaludis* (17, 29). The failure of membranes of the alanine-grown complemented cells to function in an in vitro assay system raises the possibility of whether PibD is even stably associated with the cytoplasmic membrane at all. Unfortunately, it was not possible to detect the His-tagged PibD in vivo by immunoblotting to determine its cellular location. The molecular weight of the processed flagellins, however, is consistent with their being fully glycosylated, an important point since it is known that attachment of an N-linked glycan of at least two sugars is necessary for the flagellins to assemble into filaments in *Methanococcus* (11).

Interest in archaeal protein secretion has resulted in studies of various important aspects, including the roles of the Sec and Tat pathways (16, 27), the mechanism of action of the various signal peptidases (15, 26), numerous attempts to analyze and define archaeal signal peptides (2, 5, 8), and examination of the archaeal secretome (14, 28). Still, the best studied of the archaeal signal peptidases are the type IV prepilin-like peptidases and their role in assembly of archaeal appendages like flagella and pili. The data presented in this paper indicate a difference in the minimum signal peptide length that can be processed by the only two TFPP that have been studied in any depth. This difference is significant since it places a limit on the number of potential substrates that may be processed by the enzymes of the two organisms, with the *Sulfolobus* enzyme being more flexible in its signal peptide length requirement. Coupled to its already demonstrated greater flexibility in amino acid substitutions at critical positions near the cleavage site, these findings indicate a far greater number of potential substrates for PibD than that available to FlaK. Which of these two well-studied enzymes represents the norm in *Archaea* and which the exception will have to await study of additional archaeal TFPP enzymes.

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