Identification of an Additional Minor Pilin Essential for Piliation in the Archaeon Methanococcus maripaludis

Divya B. Nair¹, Daniel K. C. Chung¹, James Schneider¹, Kaoru Uchida², Shin-Ichi Aizawa², Ken F. Jarrell¹*

¹Department of Biomedical and Molecular Sciences, Queen’s University, Kingston, Ontario, Canada, ²Department of Life Sciences, Prefectural University of Hiroshima, 562 Nanatsuka, Shobara, Hiroshima, Japan

Abstract

Methanococcus maripaludis is an archaeon with two studied surface appendages, archaella and type IV-like pili. Previously, the major structural pilin was identified as MMP1685 and three additional proteins were designated as minor pilins (EpdB, EpdB and EpdC). All of the proteins are likely processed by the pilin-specific prepilin peptidase EppA. Six other genes were identified earlier as likely encoding pilin proteins processed also by EppA. In this study, each of the six genes (mmp0526, mmp0600, mmp0601, mmp0709, mmp0903 and mmp1283) was deleted and the mutants examined by electron microscopy to determine their essentiality for pili formation. While mRNA transcripts of all genes were detected by RT-PCR, only the deletion of mmp1283 led to nonpiliated cells. This strain could be complemented back to a piliated state by supplying a wildtype copy of the mmp1283 gene in trans. This study adds to the complexity of the type IV pili system in M. maripaludis and raises questions about the functions of the remaining five pilin-like genes and whether M. maripaludis under other growth conditions may be able to assemble additional pili-like structures.

Introduction

Type IV pili are a very common type of surface appendage found in a variety of Gram-negative and Gram positive bacteria, as well as some members of the Domain Archaea [1–6]. They are involved in a wide variety of processes including adherence, aggregation, DNA transfer in transformation and conjugation, biofilm formation, electron transfer and a type of surface motility termed twitching [5–8]. The core components of a type IV pili system include structural proteins with class 3 signal peptides, a prepilin signal peptidase, one or more ATPases and a conserved membrane (platform) protein [5,9,10]. One ATPase powers the incorporation of new subunits into the growing filament while in many cases, the presence of a second, depolymerizing ATPase acts to remove subunits from the structure. The combined activities of the two ATPases result in extension and retraction of the pili, leading to the twitching motility associated with type IV pili in many bacteria [11]. The conserved inner membrane or platform protein is considered to interact with the ATPase(s) and form an export complex for the structural proteins and to be involved in both pilus assembly and disassembly [10]. In addition to these conserved components, type IV pili systems in different organisms often have other components whose role in pilus assembly and function remains unknown [6].

The structural subunits of the type IV pili consist of a major pilin and typically several other pilins, termed minor pilins due to their much lower abundance, all synthesized initially with class 3 signal peptides that are specifically processed by the prepilin peptidase [12,13]. Minor pilins have been shown to be necessary for pilus formation in several different systems [14–17] and they have been detected in sheared pili samples of N. gonorrhoeae and P. aeruginosa [16,18], although other roles for minor pilins as activators of pilus assembly without incorporation into the structure have also been proposed in other systems [19,20]. In some type IV pili systems, evidence for a minor pilus constituent acting as a specific adhesin has been presented [21–24].

Archaea are known to use the type IV pili-like model to assemble numerous surface structures [2,5,23,26] including type IV-like pili [25,26,29–32], the bindosome for substrate uptake in Sulfolobus solfataricus [25,33,34], likely the unusual Ihb670 fibres of Ignicoccus hospitalis [53,36] and the best studied example, namely the archaellum [25,28,37–40]. The name “archaellum” has been proposed to replace the term “archaeal flagellum” [27] since the archaellum structure, while involved in swimming as well as other functions, is not homologous to the bacterial flagellum and is related instead to type IV pili in structure and likely assembly [28,41,42]. This proposal is still under discussion in the scientific community and further arguments, both pro and con, have been presented [43,44].

Recent studies in Methanococcus, Haloferax and especially Sulfolobus have been devoted specifically to the study of the type IV-like pili [29–31,43–45]. Sulfolobus has been shown to produce two different type IV pili structures. One, called UV-inducible type IV pili (Ups pili; [29,47]), is widespread throughout the Sulfolobales while the second called archaecal adhesive pili (Aap pili) is limited so far to S. acidocaldarius [25,30,49]. Ups pili are upregulated under conditions that lead to DNA double stranded breaks such as UV light and their formation leads to cell aggregation that promotes DNA exchange that might help in repairing the DNA damage [29,47].
On the other hand, Aap pili are the most abundant surface appendage observed on *S. acidocaldarius* under normal growth conditions in nutrient rich medium [25]. Aap pilis are adhesion structures primarily but they also influence biofilms, promoting the formation of tower-like structures [49]. The loci identified as encoding the biosynthesis of both Aap pili and Ups pili were shown to consist of only five genes [2,29,30]. In each case, there are genes for two prepolis, a single pilin assembly ATPase, the conserved pilus membrane/platform protein and one additional gene in each operon that has an unknown function. In the Aap system, AapB appears to be the major pilin and AapA the minor pilin [30]. Mutational studies demonstrated that all five *aap* genes were necessary for pili formation [30]. In the Ups system, deletion of the gene encoding the ATPase (*upsE*) eliminated UV-induced aggregation [29]. The assignment of UpsA or UpsB proteins as the major or minor pilins has not been reported.

In *Haloflexas volcanii*, six novel type IV pilins termed PilA(1–6) involved in adhesion have been recently studied [48]. Each protein contains a highly conserved domain of unknown function (Duf1628) at their N-terminus and all are processed by PilD. Strains carrying deletions of up to five of the six pilin genes are still able to adhere while a mutant with all six genes deleted cannot. Complementation of the strain deleted for all six pilin genes with any single pilin gene results in cells that have functional pilus.

In *Methanococcus maripaludis*, an in-silico study by Szabo et al [32] identified the existence of a type IV pilin-like locus, consisting of 11 potential genes, including three encoding prepolis-like proteins and one encoding a prepilin peptidase. Subsequent genetic studies demonstrated that two of the pilin genes (*epdB* and *epdC*) were essential for pilin formation while deletion of the third (*epdA*) resulted in cells with a reduced number of pilis [31]. Unexpectedly, none of these genes encoded the major structural protein which was revealed by mass spectrometry of purified pili samples to be MMP1685, located at a distant locus [31]. Several other genes in the 11 gene operon are also essential for piliation (Nair et al. submitted) [50]. Aap pili [30]. Mutational studies demonstrated that all five *aap* genes were necessary for pili formation [30]. In the Ups system, deletion of the gene encoding the ATPase (*upsE*) eliminated UV-induced aggregation [29]. The assignment of UpsA or UpsB proteins as the major or minor pilins has not been reported.

In *Haloflexas volcanii*, six novel type IV pilins termed PilA(1–6) involved in adhesion have been recently studied [48]. Each protein contains a highly conserved domain of unknown function (Duf1628) at their N-terminus and all are processed by PilD. Strains carrying deletions of up to five of the six pilin genes are still able to adhere while a mutant with all six genes deleted cannot. Complementation of the strain deleted for all six pilin genes with any single pilin gene results in cells that have functional pilus.

In *Methanococcus maripaludis*, an in-silico study by Szabo et al [32] identified the existence of a type IV pilin-like locus, consisting of 11 potential genes, including three encoding prepolis-like proteins and one encoding a prepilin peptidase. Subsequent genetic studies demonstrated that two of the pilin genes (*epdB* and *epdC*) were essential for pilin formation while deletion of the third (*epdA*) resulted in cells with a reduced number of pilis [31]. Unexpectedly, none of these genes encoded the major structural protein which was revealed by mass spectrometry of purified pili samples to be MMP1685, located at a distant locus [31]. Several other genes in the 11 gene operon are also essential for piliation (Nair et al. submitted) [50]. Aap pili [30]. Mutational studies demonstrated that all five *aap* genes were necessary for pili formation [30]. In the Ups system, deletion of the gene encoding the ATPase (*upsE*) eliminated UV-induced aggregation [29]. The assignment of UpsA or UpsB proteins as the major or minor pilins has not been reported.

In *Haloflexas volcanii*, six novel type IV pilins termed PilA(1–6) involved in adhesion have been recently studied [48]. Each protein contains a highly conserved domain of unknown function (Duf1628) at their N-terminus and all are processed by PilD. Strains carrying deletions of up to five of the six pilin genes are still able to adhere while a mutant with all six genes deleted cannot. Complementation of the strain deleted for all six pilin genes with any single pilin gene results in cells that have functional pilus.

In *Methanococcus maripaludis*, an in-silico study by Szabo et al [32] identified the existence of a type IV pilin-like locus, consisting of 11 potential genes, including three encoding prepolis-like proteins and one encoding a prepilin peptidase. Subsequent genetic studies demonstrated that two of the pilin genes (*epdB* and *epdC*) were essential for pilin formation while deletion of the third (*epdA*) resulted in cells with a reduced number of pilis [31]. Unexpectedly, none of these genes encoded the major structural protein which was revealed by mass spectrometry of purified pili samples to be MMP1685, located at a distant locus [31]. Several other genes in the 11 gene operon are also essential for piliation (Nair et al. submitted) [50]. Aap pili [30]. Mutational studies demonstrated that all five *aap* genes were necessary for pili formation [30]. In the Ups system, deletion of the gene encoding the ATPase (*upsE*) eliminated UV-induced aggregation [29]. The assignment of UpsA or UpsB proteins as the major or minor pilins has not been reported.

In *Haloflexas volcanii*, six novel type IV pilins termed PilA(1–6) involved in adhesion have been recently studied [48]. Each protein contains a highly conserved domain of unknown function (Duf1628) at their N-terminus and all are processed by PilD. Strains carrying deletions of up to five of the six pilin genes are still able to adhere while a mutant with all six genes deleted cannot. Complementation of the strain deleted for all six pilin genes with any single pilin gene results in cells that have functional pilus.

In *Methanococcus maripaludis*, an in-silico study by Szabo et al [32] identified the existence of a type IV pilin-like locus, consisting of 11 potential genes, including three encoding prepolis-like proteins and one encoding a prepilin peptidase. Subsequent genetic studies demonstrated that two of the pilin genes (*epdB* and *epdC*) were essential for pilin formation while deletion of the third (*epdA*) resulted in cells with a reduced number of pilis [31]. Unexpectedly, none of these genes encoded the major structural protein which was revealed by mass spectrometry of purified pili samples to be MMP1685, located at a distant locus [31]. Several other genes in the 11 gene operon are also essential for piliation (Nair et al. submitted) [50]. Aap pili [30]. Mutational studies demonstrated that all five *aap* genes were necessary for pili formation [30]. In the Ups system, deletion of the gene encoding the ATPase (*upsE*) eliminated UV-induced aggregation [29]. The assignment of UpsA or UpsB proteins as the major or minor pilins has not been reported.

In *Haloflexas volcanii*, six novel type IV pilins termed PilA(1–6) involved in adhesion have been recently studied [48]. Each protein contains a highly conserved domain of unknown function (Duf1628) at their N-terminus and all are processed by PilD. Strains carrying deletions of up to five of the six pilin genes are still able to adhere while a mutant with all six genes deleted cannot. Complementation of the strain deleted for all six pilin genes with any single pilin gene results in cells that have functional pilus.

In *Methanococcus maripaludis*, an in-silico study by Szabo et al [32] identified the existence of a type IV pilin-like locus, consisting of 11 potential genes, including three encoding prepolis-like proteins and one encoding a prepilin peptidase. Subsequent genetic studies demonstrated that two of the pilin genes (*epdB* and *epdC*) were essential for pilin formation while deletion of the third (*epdA*) resulted in cells with a reduced number of pilis [31]. Unexpectedly, none of these genes encoded the major structural protein which was revealed by mass spectrometry of purified pili samples to be MMP1685, located at a distant locus [31]. Several other genes in the 11 gene operon are also essential for piliation (Nair et al. submitted) [50]. Aap pili [30]. Mutational studies demonstrated that all five *aap* genes were necessary for pili formation [30]. In the Ups system, deletion of the gene encoding the ATPase (*upsE*) eliminated UV-induced aggregation [29]. The assignment of UpsA or UpsB proteins as the major or minor pilins has not been reported.
### Table 1. Primers used in this study.

| Technique and primer name | Sequence | Restriction sites |
|---------------------------|----------|-------------------|
| **RT-PCR**                |          |                   |
| mmp0528_ RT_ for          | 5'-AATGGAATGGGAAATCTTGTTTG |                   |
| mmp0528_ RT_ rev          | 5'-AACGTTTAAAGGGCATTACTCG |
| mmp0600_ RT_ for          | 5'-GAGACATATTCGGAGCTTGG |                   |
| mmp0600_ RT_ rev          | 5'-GTGATGTTCTAGTATATGGAAGC |                   |
| mmp0601_ RT_ for          | 5'-GGAACGATATTCGGGCGACACATG |                   |
| mmp0601_ RT_ rev          | 5'-CTGGGTGGAACACTATGAGTAAGG |                   |
| mmp0709_ RT_ for          | 5'-GAGACATCTGCTGTTAATGAGTTCAGG |                   |
| mmp0709_ RT_ rev          | 5'-CGTAAATGTACGTGCTCCAGCAGGTC |                   |
| mmp0903_ RT_ for          | 5'-CCGAGATATTCGGGCGACACATG |                   |
| mmp0903_ RT_ rev          | 5'-CTGCACTGTATATACGCGGACAG |                   |
| mmp1283_ RT_ for          | 5'-GCTGGTCTCTGGCGTACTACAG |                   |
| mmp1283_ RT_ rev          | 5'-AAGCATCTTATGCTTATTGTGAG |                   |
| **Screening of deletion mutants** |          |                   |
| mmp0528_ seq _ for        | 5'-GGATCATGGGGAGATGACCC |                   |
| mmp0528_ seq _ rev        | 5'-GTGCACGGCATACATTCGTG |                   |
| mmp0600_ seq _ rev        | 5'-TACTGGCGACTATTATACATTGG |                   |
| mmp0600_ seq _ rev        | 5'-GAAATAGTATTTGGCTCACTAGTTCG |                   |
| mmp0601_ seq _ for        | 5'-TGACTACGTGATTATTGGAAGAG |                   |
| mmp0601_ seq _ rev        | 5'-GTATAATTCTATCGAATCTGG |                   |
| mmp0709_ seq _ for        | 5'-GAGATATGCCTCATTGGGAGTC |                   |
| mmp0709_ seq _ rev        | 5'-GTTCCGTACAGTAAATTACC |                   |
| mmp0903_ seq _ for        | 5'-CCGAAACTTAAATTTAGGAGG |                   |
| mmp0903_ seq _ rev        | 5'-CCGAGAATTTGACCTGTGTCG |                   |
| mmp1283_ seq _ for        | 5'-GAAGGTGTATTGGTTATAC |                   |
| mmp1283_ seq _ rev        | 5'-GAGACATGTTACGTCAATCG |                   |
| **In-frame deletions/plasmids** |          |                   |
| mmp0528_P1                | 5'-CGGATCCGGATATTACGGAAAGAAGT | BamH1 |
| mmp0528_P2                | 5'-TTGGCGCGCCATGTTTGGCGGAAATATTCTTG | AscI |
| mmp0528_P3                | 5'-TTGGCGCGCCACACAGATTCCCAATCCC | AscI |
| mmp0528_P4                | 5'-CGCATCCACATTTCTGCACAATCTCG | BamH1 |
| mmp0600_P1                | 5'-GGGATCCGGCAGAAGAAGAATACG | BamH1 |
| mmp0600_P2                | 5'-TTGGCGCGCTACCTGCGAAGAAGAATACG | AscI |
| mmp0600_P3                | 5'-TTGGCGCGCCACAATCGGGAAGAATACG | AscI |
| mmp0600_P4                | 5'-CGGATCCCGACTGAGAAGAATACG | BamH1 |
| mmp0601_P1                | 5'-TTGGCGCGCCACTGAGAAGAATACG | AscI |
| mmp0601_P2                | 5'-TTGGCGCGCCACTGAGAAGAATACG | AscI |
| mmp0601_P3                | 5'-TTGGCGCGCCACTGAGAAGAATACG | BamH1 |
| mmp0601_P4                | 5'-TTGGCGCGCCACACTGACGAAGAATACG | AscI |
| mmp0709_P1                | 5'-GGGATCCCGAAATCGGAATGTTG | BamH1 |
| mmp0709_P2                | 5'-GGGATCCCGGATTTGCGGAAACG | AscI |
| mmp0709_P3                | 5'-TTGGCGCGCCACTGAGAAGAATACG | AscI |
| mmp0709_P4                | 5'-GGGATCCCGGAATCGGAATGTTG | BamH1 |
| mmp0903_P1                | 5'-GGGATCCCGGAATCGGAATGTTG | BamH1 |
| mmp0903_P2                | 5'-GGGATCCCGGAATCGGAATGTTG | AscI |
| mmp0903_P3                | 5'-GTGGCGCGCCAGAAGAATACG | AscI |
| mmp0903_P4                | 5'-GGGATCCCGGAATCGGAATGTTG | BamH1 |
| mmp1283_P1                | 5'-GGGATCCCGGAATCGGAATGTTG | BamH1 |
| mmp1283_P2                | 5'-GGGATCCCGGAATCGGAATGTTG | AscI |
Complementation of the Δmmp1283 gene deletion were done in pWLG40 [55] in which the complementing gene is under the control of the constitutive, strong hmv promoter [31,56,57]. For this, the mmp1283 gene was amplified using the forward and reverse complementation primers (Table 1) containing added AscI and BamHI restriction sites, respectively. The PCR product was digested with AscI and BamHI and cloned into pWLG40, to generate pKJ1007. This plasmid was transformed into M. maripaludis Δmmp1283 using puromycin for selection.

Electron Microscopy
Overnight cultures were washed with 50 mM MgSO4, and negatively stained with 2% phosphotungstic acid. Cells were examined on Formvar-coated gold grids and imaged under a Hitachi 7000 electron microscope operating at an accelerating voltage of 75 kV.

Results
Using the FlaFind program, Szabo et al. 2007 [32] identified 14 proteins in M. maripaludis that had class 3 signal peptides characteristic of archaellins and bacterial type IV pilins. Of these 14 genes, three were previously identified as archaellins [57] and several were already shown to be involved in pili formation [31]. The latter included epdA, epdB and epdC as well as the major pilin gene mmp1685. One of the 14 genes is a NAD+ synthase-related protein while the remaining six genes encode type IV pilin-like proteins with a DUF361 Pfam domain. The six pilin-like proteins were predicted to be processed by EppA, a prepilin peptidase that was already shown to process the DUF361 domain-containing pilins EpdA and EpdC [32]. The six genes under study here as encoding potential pili structural proteins are mmp0528, mmp0600, mmp0601, mmp0709, mmp0903 and mmp1283. All of these proteins have a class 3 signal peptide of 5–13 amino acids ending with a glycine, a conserved +5 glutamic acid, and a conserved +1 glutamine (Figure 1), which may be required for EppA processing [32]. Of the six genes, mmp0600 and mmp0601 appear to be in an operon while the four remaining genes are not. Mmp0528, Mmp0903 and Mmp1283 are very small proteins of 67–76 amino acids in length (54–63 amino acids after signal peptide removal), almost identical to the size of the major structural pilin, Mmp1685 which is 74 amino acids in length (62 amino acids after signal peptide removal). The minor pilins already identified (EpdA, EpdB and EpdC) are about twice as long (130–156 amino acids). Mmp0600, Mmp0601 and Mmp0709 are much larger proteins (200–299 amino acids). Mmp1685 is known to be a glycoprotein with an attached N-linked pentasaccharide identical in structure to the tetrasaccharide identified attached to archaellins [58] but with an additional hexose attached as a branch to the linking sugar N-acetyl-galactosamine [31]. Analysis of the sequence of the 6 putative minor pilins identified 1–6 N-glycosylation sequons (N-X-S/T, where X is not proline) which indicates that all of these proteins could also be N-glycosylated (Figure 1).

As a first step to determining that the 6 putative minor pilin genes correspond to true genes, evidence for an mRNA transcript of each gene was sought using RT-PCR since detection of transcript provides support that an ORF indeed encodes a true protein [59]. RT-PCR was performed on isolated total RNA using primers that would amplify an internal fragment of each gene. In all cases, a PCR product was obtained of the predicted size only when the RNA was subjected to a reverse transcription step and not from the RNA sample itself (Figure 2), indicating that the product arose from cDNA and not contaminating genomic DNA present in the RNA preparation. The product of the RT-PCR was, in each case, identical in size to that obtained using genomic DNA as template. Sequencing of each PCR product confirmed
their identities. Thus, all 6 putative pilin genes were transcribed under standard growth conditions in Balch medium III at 35° C.

Each of the 6 genes was then targeted for deletion. The parent strain for these deletions was M. maripaludis DflaK [31]. This strain is deleted for flaK which encoded the prepilin peptidase essential for signal peptide removal from archaellins [60,61]. Without this processing, the cells cannot assemble archaella so that the only surface structures remaining are pili. This makes analysis of effects on piliation by specific gene deletions easier to visualize.

Transformants were screened using a PCR method with whole cells as template and primers that would amplify across the targeted genes. Successful deletion of each gene would result in a smaller PCR amplification product, whose size can be predicted from the site of the primers used in the PCR. Mutants carrying a deletion of each of the 6 putative minor pilin genes were obtained (Figure 3).

To investigate whether any of the targeted genes played an essential role in piliation, all mutants were examined by electron microscopy for the presence and abundance of pili. In bacterial type IV pili systems usually there is one major pilin and a number of minor pilins [5,20]. The major pilin, as well as three minor pilins, were already identified in M. maripaludis so it seemed...
unlikely that all six putative minor pilin genes studied here would be involved in assembly of the MMP1685 pili as this would result in a total of ten different structural proteins. The electron microscopic examination of the various mutants supported this contention as only the strain carrying the deletion of mmp1283 was nonpiliated (Figure 4). Strains with deletions in mmp0528, mmp0600, mmp0601, mmp0709 and mmp0903 were all piliated to the extent of the parent M. maripaludis ΔflaK cells (compare pilation here to that seen in M. maripaludis ΔflaK cells in Figure 5). In the case of the mmp1283 deletion strain, complementation with a wildtype version of mmp1283 supplied in trans restored the cells to a piliation state comparable to M. maripaludis ΔflaK cells (Figure 5).

Discussion

Methanococcus maripaludis is known to have at least two surface appendages that are assembled in a bacterial type IV pili mode, namely archaella and type IV-like pili [3,26,62]. Unusual for Archaea is that the processing of the structural subunits, i.e. archaellins and pilins, in M. maripaludis has been demonstrated to occur through the actions of two different prepilin peptide-like enzymes whose substrate specificities do not overlap. FlaK processes only archaellins and EppA only pilins [32,60,63]. In other studied Archaea, a single enzyme designated PibD is thought to remove the signal peptide from all pilin-like substrates, including archaellins [25,64,65]. As more Archaea are studied, it seems likely that the division of labor in processing prepilin-like substrates by two separate prepilin peptide-like enzymes reported so far only in M. maripaludis may be found in other members of the domain. It has been reported that several members of the Eurarchaeota, mainly Methanococcales as well as Pyrococcus and Thermococcus species harbor both flaK and eppA homologs in their genomes [32]. There are also a limited number of Eurarchaeota that have been reported to possess more than one copy of flaK [66] but the roles and substrates of these potential prepilin peptidases, some found in species reported to be non-archaellated cells, has not been studied.

Previous studies have demonstrated roles for four pilins in the biosynthesis of the M. maripaludis pili. Genes encoding three minor pilins, EpdA, EpdB and EpdC are found in a single large gene cluster [32] that also includes EppA and several other genes shown to be essential for pilus formation [Nair et al., submitted]. Deletions of the genes for the three pilins result in either completely nonpiliated cells or cells in which the number of pili is significantly reduced [31]. The major structural protein was identified as MMP1685 and deletion of mmp1685 led to nonpiliated cells. Interestingly, MMP1685 had been previously identified by bioinformatics analysis as a predicted substrate for EppA [32]. In addition, that study also predicted that six other genes encoded pilin-like proteins likely to be EppA substrates. In this report, deletions were created in all six genes to investigate the potential role of the encoded pilin-like proteins in the biosynthesis of the surface pili of M. maripaludis.

Three of the pilin-like proteins MMP0528, MMP0903 and MMP1283 are of similar size to the previously identified major pilin structural protein MMP1685 [31] while the other three (MMP0600, MMP0601 and MMP0709) are much larger. The smaller pilin sizes are typical lengths for type IV pilins of the Flp (Tad) class and the presence of +1 glutamine is also common in Gram positive Flp pilins [1,5,6]. M. maripaludis pilins, however, lack the +6 tyrosine and so called Flp motif of Flp pilins. All six pilin-like proteins possess a +5 glutamic acid, conserved in most bacterial type IV pilins [5] but absent in the pilins of both Sulfolobus [2,30] and Haloferax [48]. Examination of mutant strains carrying deletions of each of the targeted genes by electron microscopy indicated that only mmp1283 was essential for piliation as all other deletion strains had similar numbers of pili per cell as wildtype cells. A piliated state could be restored to the Δmmp1283 strain by supplying a wildtype copy of the gene in trans under the control of a constitutive haw promoter. Like the major pilin MMP1685 and all the previously identified minor pilins (EpdA, EpdB, and EpdC), MMP1283 carries an amino acid sequon necessary for N-linked glycan attachment, suggesting that MMP1283 may be modified by the pentasaccharide found attached to MMP1685 [31]. While the M. maripaludis major pilins are modified with the N-linked glycan, this posttranslational modification is not needed for pilus formation as all pilin-like proteins are formed even in an aglB mutant that is missing the oligosaccharyltransferase necessary to transfer the glycan from its lipid carrier to the protein target [67]. It is not yet known if these assembled pili, however, are functional in surface attachment, the only known function attributed to the pili [62].

The function of the other pilin-like genes shown not to be essential for the MMP1685 pili is unknown but several possibilities exist. They could still be involved in the MMP1685 pili structure but dispensable proteins. In Neisseria meningitidis type IV pili, five different pilin proteins are necessary for piliation while three others that are normally incorporated into the pilus are dispensable for piliation formation but play important roles in function. Interestingly, these three minor pilins (PilX, ComP and PilV) all have different

Figure 3. Confirmation of the deletion of each of the six pilin-like genes. PCR reactions used whole cells of the wildtype or the deletion strains as template with gene specific primers. In the case of each gene, the first lane is the PCR product obtained with wildtype cells as template and the second lane is the PCR product obtained with the deletion strain for that gene as template. In all cases a smaller PCR product is obtained for the deletion strain and the predicted sizes of the amplicons were obtained. doi:10.1371/journal.pone.0083961.g003
functions [68]. Alternatively, the pilin-like proteins could be structural proteins of an entirely separate pilus-like structure. It is known in the thermoacidophilic archaeon *Sulfolobus acidocaldarius* that two different types of type IV pili are made [25,30,47] with one type (Ups pili) only made after UV induction or other DNA damaging treatment [29,47]. Perhaps, under still undefined growth conditions or stress, *M. maripaludis* has the capacity to assemble a novel pilus type composed of one or more of the remaining pilin-like proteins that currently have no function.

Although mRNA transcripts were detected for all five of the other pilin-like genes by RT-PCR, it is possible that posttranscriptional regulation mechanisms prevent pilin protein synthesis. The regulation of archaella assembly, for example, in *S. acidocaldarius* seems to involve both transcriptional and posttranscriptional control [25,69]. Alternatively, in the case of the five pilin-like proteins for which deletion did not affect formation of MMP1685 pili, other key components essential for pili formation from these pilin-like proteins may not be made under tested growth conditions.

**Figure 4.** Electron micrographs of strains carrying deletions of each of the six pilin-like genes. An enlargement of a portion of each mutant cell is presented below the intact cell to enhance visualization of pili. Arrows indicate pili on the cell surface. Only the *M. maripaludis* Δmmp1283 strain is nonpiliated. Bar, 0.5 μm. doi:10.1371/journal.pone.0083961.g004
conditions, even though the pilins themselves are made. In the case of *S. acidocaldarius*, archeaella synthesis is induced under tryptone starvation conditions even though archeaella core proteins are constitutively produced. This is because the major structural protein (the archeaellin FlaB) is only made under starvation conditions [25].

A third possibility is that *M. maripaludis* makes a very short pilus-like structure from one or more of these proteins that has gone undetected by electron microscopy. In *Sulfolobus solfataricus*, it is known that sugar binding proteins are pilin-like glycoproteins that form a macromolecular cell-surface-associated structure [25,33] that may form a short pilus-like structure that extends only from the cytoplasmic membrane to the S-layer [3]. The putative bindosome pilus-like structure has never been observed in electron microscopic studies. Type two secretion systems in bacteria use type IV pilin-like proteins to produce a very short pilus-like piston (pseudopilus) proposed to push exoproteins through an outer membrane channel [70]. This pseudopilus would extend only from the cytoplasmic membrane to the outer membrane and likely be dynamic in nature. While *M. maripaludis* does not utilize sugars as substrates or possess a type II secretion system, there may be other functions in the cells that may require such a short type IV pilus-like structure. The recent identification of putative diverse type IV pilin in a variety of Gram positive bacteria using a program called PilFind [1] suggest that all possible functions for these structures have not likely been identified yet.

In bacteria, it is not unusual for type IV pilin to be composed of a major pilin and multiple minor pilins. For example, in *P. aeruginosa*, the pilus is comprised of the major pilin PilA and five minor pilins (FimU, PilV, PilW, PilX and PilE) which were all shown to be incorporated into the pilus by immunogold labelling experiments [18]. Among the studied Archaea, however, the type IV pilus loci of *M. maripaludis* appears to be considerably more complex than the two gene clusters encoding Aap and Ups pili in *Sulfolobus*. In both the Aap and Ups pili systems, there appear to be only two pilin genes and they are encoded along with the conserved ATPase and membrane component genes. Interestingly, in the case of Aap pilins of *S. acidocaldarius* and Ups pili of *S. solfataricus*, the lengths (138–168 amino acids with signal peptides) are much larger than seen with MMP1685. In the case of the six recently described *Hfx. volcanii* pilins, none are co-transcribed with the pilus ATPase and conserved membrane protein genes [48]. The *M. maripaludis* pili genes are known to lie now in at least four separate locales around the chromosome. One major operon encoding EppA, EpdA, EpdB and EpdC along with other essential genes has already been analyzed to some extent [31,32]. Furthermore, there is a separate locus encoding the ATPase and two membrane protein components (Nair et al., submitted) as well as the major pilin subunit MMP1685 [31] and now, in this report, the minor pilin MMP1283. It is not yet known if type IV pili formation is constitutive in *M. maripaludis* or whether it can be induced or repressed under specific environmental circumstances such as attachment or planktonic conditions, as is observed in bacterial type IV pilus systems [5]. If pili formation is not constitutive then the cells must regulate transcription of several essential gene clusters located at some distance from each other. Regulation of minor pilin expression has not been well studied even in bacteria. In type IVb systems where minor pilins are clustered with other components of the pilus system, they are likely co-regulated with them. However, in type IVa systems, minor pilins are often unlinked to other pilus component genes, as found in *M. maripaludis*, and they can be differentially regulated, sometimes by two-component systems [5,6,71].

![Figure 5. Electron micrographs showing that complementation restores piliation to the *M. maripaludis* Δmmp1283 strain. The *M. maripaludis* ΔflaK strain (non-archaellated) used as the parent for the pilin gene deletion studies is shown for comparison. An enlargement of a portion of each mutant cell is presented below the intact cell to enhance visualization of pili. The *M. maripaludis* Δmmp1283 strain was complemented with a plasmid-borne wildtype version of the mmp1283 gene under the control of the constitutive hmv promoter. Bar, 0.5 μm. doi:10.1371/journal.pone.0083961.g005](image-url)
In *Sulfolobus* species, studies on the regulation of pilin have already been initiated, with intriguing findings reported. In *S. acidocaldarius*, a two component regulatory system (ArnA and ArnB) was found to repress archeaellla expression. Interestingly, overproduction of ArnA also resulted in a strong enhancement of Aap pilus production suggesting there is a regulation of the two surface organelles that involves cross-talk between the two systems [69]. Recently, the product of an Lrs14 regulator gene sac0446, was shown to bind to promoters of both archeaellla (*fla*) genes and aap pilin genes and result in an upregulation of *aap* genes and downregulation of *fla* genes, again showing that regulation of different surface structures in this archeaon is connected [46].

This report adds to our knowledge about the complexity of the type IV-like pili in *M. maripaludis* by identifying a fourth minor pilin that is essential for pilation. Unlike other archeal systems, pilation in *M. maripaludis* requires a separate pilin-specific signal peptidase, two conserved membrane (platform) proteins, four minor pilins and additional novel essential proteins [31,32]. This report also eliminates five other pilin-like proteins as playing an essential role in MMP1653 pilum formation and hints that they may form additional type IV-like pilum surface structures under appropriate growth conditions.

### Author Contributions

Conceived and designed the experiments: KFJ DBN. Performed the experiments: DBN DKCC JS KU SA KFJ. Analyzed the data: DBN SA KFJ. Contributed reagents/materials/analysis tools: KFJ SA. Wrote the paper: DBN KFJ.

### References

1. Imam S, Chen Z, Roos DS, Pohlschroder M (2011) Identification of surprisingly diverse type IV pili, across a broad range of gram-positive bacteria. PloS one 6: e28919.

2. Pohlschroder M, Ghosh A, Tripepi M, Albers SV (2011) Archaeal type IV pilus-like structures—evolutionarily conserved prokaryotic surface organelles. Curr Opin Microbiol 14: 1–7.

3. Ng SYM, Zolghadr B, Driessen AJM, Albers SV, JarrellKF (2008) Cell surface structures of archaea. J Bacteriol 190: 6039–6047.

4. Pelicic V (2008) Type IV pilus: E pluribus unum? Mol Microbiol 68: 827–837.

5. Gilmer CL, Neumann Y, Burrows LL (2012) Type IV pili: proteins: Versatile molecular modules. Microbiol Mol Biol Rev 76: 740–772.

6. Burrows LL (2012) *Pseudomonas aeruginosa* adhesin. Cell Microbiol 11: 1173.

7. Alm RA, Hallinan JP, Watson AA, Mattick JS (1996) Fimbrial biogenesis genes, again showing that regulation of genes, again showing that regulation of...
48. Esquivel RN, Xu R, Pohlschroder M (2013) Novel archaeal adhesion pilins with a conserved N terminus. J Bacteriol 17: 3808–3818.
49. Henche AL, Koerdt A, Ghosh A, Albers S (2012) Influence of cell surface structures on crenarchaeal biofilm formation using a thermostable green fluorescent protein. Environ Microbiol 14: 779–793.
50. Moore BC, Leigh JA (2005) Markerless mutagenesis in Methanococcus maripaludis demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. J Bacteriol 187: 972–979.
51. Ng SY, VanDyke DJ, Chaban B, Wu J, Nosaka Y, et al. (2009) Different minimal signal peptide lengths recognized by the archaeal prepilin-like peptidases FlaK and PibD. J Bacteriol 191: 44–50.
52. Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: Reevaluation of a unique biological group. Microbiol Rev 43: 260–296.
53. VanDyke DJ, Wu J, Ng SY, Kanbe M, Chaban B, et al. (2008) Identification of putative acetyltransferase gene, MMP0350, which affects proper assembly of both flagella and pili in the archaeon Methanococcus maripaludis. J Bacteriol 190: 5300–5307.
54. Tumbula DL, Makula RA, Whitman WB (1994) Transformation of Methanococcus maripaludis and identification of a PstI-like restriction system. FEMS Microbiol Lett 121: 309–314.
55. Lie TJ, Leigh JA (2003) A novel repressor of nif and glnA expression in the methanogenic archaeon Methanococcus maripaludis. Mol Microbiol 47: 235–246.
56. Lie TJ, Wood GE, Leigh JA (2005) Regulation of nif expression in Methanococcus maripaludis: Roles of the euryarchaeal repressor NrpR, 2-oxoglutarate, and two operators. J Biol Chem 280: 5236–5241.
57. Chaban B, Ng SY, Kanbe M, Saltzman I, Nimmo G, et al. (2007) Systematic deletion analyses of the fla genes in the flagella operon identify several genes essential for proper assembly and function of flagella in the archaeon, Methanococcus maripaludis. Mol Microbiol 66: 596–609.
58. Kelly J, Logan SM, Jarrell KF, VanDyke DJ, Vandyke DJ, Vinogradov E (2009) A novel N-linked flagellar glycan from Methanococcus maripaludis. Carbohydr Res 344: 648–653.
59. Abu-Qarn M, Eichler J (2006) Protein N-glycosylation in archaea: Defining Haloferax volcanii genes involved in S-layer glycoprotein glycosylation. Mol Microbiol 61: 511–523.
60. Barry SL, Jarrell KF (2002) FlaK of the archaeon Methanococcus maripaludis possesses preflagellin peptidase activity. FEMS Microbiol Lett 208: 53–59.
61. Barry SL, Jarrell KF (2005) Cleavage of preflagellins by an aspartic acid signal peptide is essential for flagellation in the archaeon Methanococcus voltae. Mol Microbiol 50: 1339–1347.
62. Jarrell KF, Stark M, Nair DB, Chong IP (2011) Flagella and pili are both necessary for efficient attachment of Methanococcus maripaludis to surfaces. FEMS Microbiol Lett 319: 44–50.