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ORIGINAL PAPER

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Insertional inactivation of the *flaH* gene in the archaeon *Methanococcus voltae* results in non-flagellated cells

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Abstract The marine methanogen *Methanococcus voltae* possesses two transcriptional units that encode a total of four flagellins. Immediately downstream of the flagellin genes are a number of ORFs, some of which are cotranscribed with the flagellin genes. These putative genes have been named *flaCDEFGHIJ*, although no biochemical data has implicated them in flagellar morphogenesis. None of the *flaC-J* genes has homology to any bacterial gene, with the exception of *flaI*, which shows homology to *pilT*, a gene that encodes a nucleotide binding protein of the type IV pilus family. In this study, insertional mutations in *flaH* of *M. voltae* were identified. The mutants were non-motile and non-flagellated as determined by electron microscopy. Southern hybridization experiments confirmed the insertion of a mutagenic vector into *flaH* and indicated that two, tandem, copies of the vector were present. It is believed that insertion of the vector into *flaH* should disrupt the transcription of *flaIJ* due to polar effects. The *flaH* mutant displayed the same pattern of multiple mRNA transcripts, all originating upstream of *flaB1*, as the wild-type cells. Northern hybridization experiments failed to detect a *flaHIJ* transcript in either wild-type or mutant cells. Immunoblotting experiments indicated, however, that the mutants produced similar amounts of flagellin, FlaD and FlaE to wild-type cells. Flagellin localization experiments suggest that the *flaH* mutant is deficient in flagellin secretion and/or assembly. The mutant also displayed similar preflagellin peptidase activity to the wild-type cells, indicating that none of the genes *flaHIJ* is likely to be the gene that encodes this enzyme, which is required for cleaving the leader peptide from the preflagellins prior to their incorporation into the flagellar filament. This is the first data indicating that the *flaHIJ* gene cluster is essential for flagellation in methanogens.

Keywords *Methanococcus voltae* · Flagellum biosynthesis · Flagellum assembly · Insertional inactivation · *flaHIJ*

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Introduction

The archaeal flagellum has been shown to be a unique motility structure (Jarrell et. al. 1996b) that differs from the bacterial flagellum in both ultrastructure and composition. Archaeal flagella are thin filamentous structures (10–13 nm in diameter) that give cells the ability to swim (Alam and Oesterhelt 1984; Jarrell et al. 1996a). The filaments have been shown to rotate in halobacterial species (Marwan et al. 1991) and chemotactic behaviour has been observed in several archaeal species (Sment and Koninsky 1989; Rudolph and Oesterhelt 1996). In *Methanococcus voltae*, a marine methanogen, there are four flagellin genes. It is known that the bulk of the *M. voltae* filament is composed of two flagellins, FlaB1 and FlaB2 (Kalmokoff et al. 1988), and a third flagellin, FlaB3, has recently been located at the cell-proximal end of the filament (Bardy and Jarrell, unpublished data). The location of the fourth flagellin, FlaA, has yet to be determined. In *Halobacterium salinarum*, an extreme halophile, it was demonstrated that all five flagellin gene products (FlgA1 and A2, and FlgB1, B2 and B3) are components of the filament (Gerl et al. 1989). Mutant studies in this archaeon (Tarasov et al. 2000) suggest that the B flagellins are found at the proximal end of the filament, while the A flagellins are at the distal end.

An unexpected finding was that archaeal flagellins are made as preproteins with short leader peptides that are cleaved before the mature proteins are incorporated in

the flagellar filament (Kalmokoff and Jarrell 1991). In *M. voltae*, cleavage of the preprotein (preflagellin) is achieved by a peptidase, which is located in the membrane fraction of cells (Correia and Jarrell 2000). The fact that archaeal flagellins are initially made as preproteins that are processed suggests a mode of biosynthesis for archaeal flagella different from the bacterial model for flagellar assembly.

The hypothesis has been presented that archaeal flagellin export may occur by a type II-like secretion pathway (Bayley and Jarrell 1998). It is evident that Sec protein homologues and type II secretion homologues are present in complete genome sequences of archaeal species (Bult et al. 1996; Kawarabayasi et al. 1998). Archaeal flagellins share sequence similarity at the N-terminal region with type IV pilins of gram-negative bacteria (Faguy et al. 1994). Interestingly, type IV pili mediate a surface-dependent movement called twitching motility (Bradley 1980; Wall and Kaiser 1999). Both archaeal flagellins and type IV pilins have hydrophobic N-terminal regions and both types of proteins are made as preproteins with unusual leader sequences. Since very few studies have addressed protein secretion and cell surface assembly of proteinaceous structures in the Domain Archaea, archaeal flagellins are excellent models with which to evaluate these cellular processes.

The biochemistry and genetics of archaeal flagella have been well studied in *M. voltae*. In this methanogen, two transcriptional units that encode some of the structural components necessary for flagellum biosynthesis have been identified (Kalmokoff and Jarrell 1991) (Fig. 1). The first unit comprises a single flagellin gene, *flaA*. The second unit is made up of three more flagellin genes (*flaB1*, *flaB2* and *flaB3*) and possibly eight additional genes located immediately downstream, designated *flaCDEFGHIJ*. Transcription of the second set of genes is driven from a promoter located upstream of *flaB1*. Three different polycistronic mRNAs of 1.3, 4.2 and 5.4 kb can be identified. The longest message would be expected to end after *flaG*. No mRNAs extending into *flaH*, *flaI* or *flaJ* have been detected, calling into question their original designation as accessory flagellar genes.

The assignment of putative biological functions to the proteins encoded by these genes is hampered by the fact that almost none of the *flaC-J* genes have homologues in gene banks. The sole exception is FlaI, which shares homology with PilT, a protein involved in type IV pilus biogenesis and function (Whitchurch et al. 1990). Furthermore, both FlaH and FlaI have sequences that correspond to nucleotide binding domains, although this activity has not been demonstrated for these proteins. In the flagellated archaeal species for which sequence data are available, *flaH*, *flaI* and *flaJ* are always present and located in close proximity to flagellin genes. Due to the conservation of this group of genes in flagellated archaeal species, we hypothesized that they are likely to be involved in flagellum biosynthesis or flagellar function. In this study, we report on the insertional inactivation of *flaH* using a mutational vector developed for genetic studies in *M. voltae*, and demonstrate that the *flaH* mutants isolated are non-flagellated. This is the first demonstration that any of the *flaHIJ* genes are actually required for flagella assembly in an archaeon.

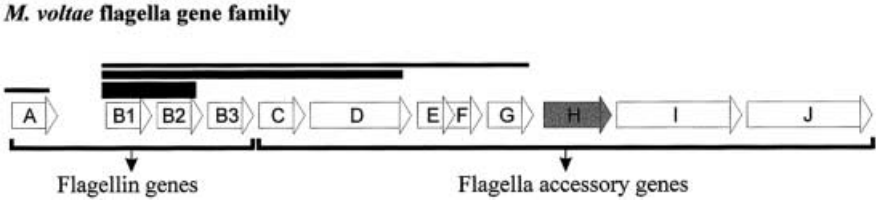

Fig. 1 Schematic representation of the flagella gene family of *M. voltae*. The lines *above* the genes represent mRNA species that have been detected for this gene family. The *thickness* of the lines above the genes indicates the relative abundance of the mRNAs, but are not intended to be quantitative

Materials and methods

Microorganisms and growth conditions

M. voltae strain PS (obtained from G. D. Sprott, National Research Council of Canada, Ottawa, Ontario) was grown in Balch medium 3 (Balch et al. 1979) at 37°C under an atmosphere of CO_2/H_2 (20:80). *Escherichia coli* (DH5α) was grown in Luria-Bertani (LB) medium (Sambrook et al. 1989) at 37°C and supplemented with 100 μg/ml ampicillin when necessary.

DNA manipulations and analyses

Insertional inactivation of the chromosomal copy of *flaH* was performed by using an internal portion of the gene cloned into a mutagenic vector. Plasmid pKJ295 (Fig. 2) was constructed by cloning an internal 560-bp fragment of *flaH*, generated by PCR, into a *pac*/pUC vector developed for genetic transformation of *M. voltae* (Berghofer and Klein 1995). In the vector, the *pac* expression unit containing the puromycin transacetylase gene of *Streptomyces alboniger* is under the control of the promoter and terminator of the *M. voltae mcr* transcriptonal unit (Gernhardt et al. 1990). PCR was performed on a MiniCycler (MJ Research, Watertown, Mass.), using *Pwo* polymerase (Roche Molecular Biochemicals, Laval, Quebec). The cycling conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 45 s, 47°C for 45 s, and 72°C for 1 min; and a final cycle with an extension time lengthened to 5 min. Plasmid pKJ201, which contains the *flaH* gene cloned as a *Nde*I-*Xho*I fragment, served as the template in the reaction. The forward primer (5′-CG*GAATTC*CATATTGAAGGTGAGG-3′) corresponding to positions 3437–3454 of *flaH* (Genbank Accession No. U97040) and the reverse primer (5′-CG*GAATTC*TGGAATGGTCCTGCAG-3′) complementary to positions 3966–3983 amplify a 560-bp internal fragment of *flaH* with terminal *Eco*RI restriction sites (italicized). This PCR fragment was purified using a PCR purification kit (Qiagen, Chatsworth, Calif.), digested with *Eco*RI and then ligated into an

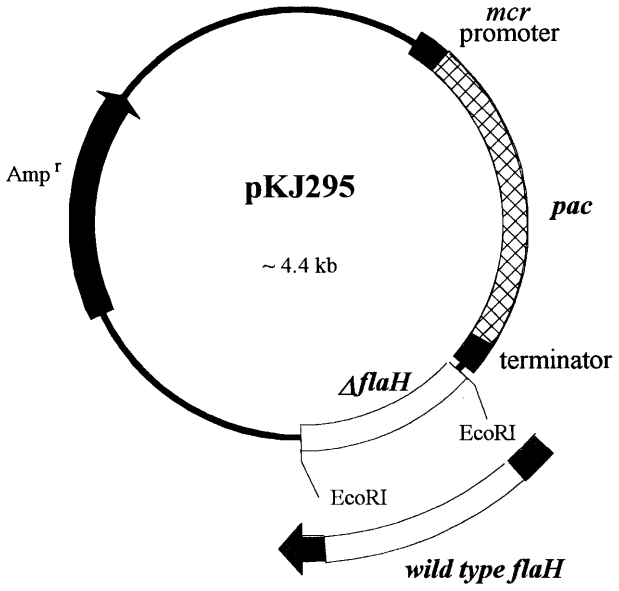

Fig. 2 Schematic diagram of pKJ295, the mutagenic vector constructed for insertional inactivation of the chromosomal copy of *M. voltae flaH*. The *mcr* promoter and terminator flank the *pac* gene. An internal *flaH* DNA fragment from *M. voltae* (*open box*) was amplified from a wild type copy of the complete gene by PCR (shown *below* the plasmid). Terminal *Eco*RI sites were incorporated during PCR, thus allowing cloning into the unique *Eco*RI site in *pac*/pUC to generate pKJ295

*Eco*RI-digested and dephosphorylated *pac*/pUC vector. The resulting constructs were transformed into competent *E. coli* cells, which were then screened for insert orientation. pKJ295 contains the PCR fragment in the same orientation as the *mcr* promoter in the plasmid.

Transformation of *M. voltae*

A liposome delivery method described for *Methanosarcina acetivorans* (Metcalf et. al. 1997) was used for transformation of *M. voltae* with some modifications. All steps were performed anaerobically using reduced buffers. DNA-containing liposomes were prepared by mixing 3 μg of plasmid DNA (in 22.5 μl of 20 mM HEPES buffer, pH 7.4) with 2.5 μl of Escort Transfection Reagent (1:1 ratio of DOPE:DOTAP; Sigma, St. Louis, Mo.) for 10 min. Ten millilitres of cells grown overnight with gentle shaking were harvested anaerobically by centrifugation at $8000 \times g$ for 10 min in Corex tubes modified to accept serum stoppers. The supernatant was removed and the cells were resuspended in 5 ml of 0.85 M sucrose. Five hundred microlitres of cells were added to the prepared DNA/liposome mixture and incubated anaerobically at room temperature for 1.5 h. Control transformations were also performed with liposomes without DNA. The cells were then added to 10 ml of Balch medium 3 (without selection) and allowed to grow overnight. The established culture was then inoculated into fresh medium containing 7.5 μg/ml puromycin to select for puromycin-resistant transformants. Streaking for isolated colonies was done on bottle plates containing Balch medium 3 [with puromycin at 7.5 μg/ml and solidified with 1.5% (w/v) agar] in an anaerobic chamber that contained 5% CO_2, 10% H_2 and 85% N_2. After streaking, the bottle plates were removed from the anaerobic chamber and the headspace was filled with CO_2/H_2 (20:80). The plates were incubated at 37°C and isolated colonies appeared after 10–14 days of incubation. Single, isolated colonies were picked and inoculated within the anaerobic chamber into Balch medium 3 containing puromycin.

RNA isolation

Total cellular RNA was isolated from late log-phase cells by using a RNeasy Mini Kit (Qiagen) with some modifications. Six millilitres of wild type or transformant *M. voltae* cell cultures were harvested anaerobically by centrifugation in modified Corex tubes at $8000 \times g$ for 10 min. The supernatant was removed and the cells were resuspended in 450 μl of a mixture of RLT (lysis buffer supplied with Qiagen RNeasy Mini Kit) and TE buffer (350 μl:100 μl). This treatment resulted in immediate cell lysis. The lysate was then processed and loaded onto spin columns as recommended by the manufacturer. Purified RNA was eluted in 45 μl of RNase-free water and treated with RQ1 RNase-free DNase (Promega, Madison, Wis.) at 37°C for 30 min. After quantifying the RNA concentration by spectrophotometry, 10 μg RNA was added to an appropriate volume of $5 \times$ gel loading buffer [this stock solution contains 16 μl of saturated bromophenol blue, 80 μl of 500 mM EDTA pH 8.0, 720 μl of 37% formaldehyde, 2 ml of 100% glycerol, 3.1 ml of formamide, 4 ml of $10 \times$ FA gel buffer (10X FA gel buffer is 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0)], heated to 65°C for 5 min and incubated on ice prior to loading onto a 1.2% agarose gel containing formaldehyde (1.8 ml of 37% formaldehyde per 100 ml gel). All solutions used for RNA isolation and analysis were made in diethylpyrocarbonate (DEPC)-treated water. RNA markers (0.24–9.5 kb RNA Ladder; Life Technologies, Gibco-BRL, Mississauga, Ontario) were used as size standards. Following electrophoresis in $1 \times$ FA gel buffer supplemented with formaldehyde (20 ml of 37% formaldehyde per litre of $1 \times$ FA gel buffer), RNA was transferred to nylon membranes (Roche Molecular Biochemicals) using a capillary transfer technique (Sambrook et al. 1989).

Southern and Northern hybridization analyses

Chromosomal DNA was isolated from wild-type and transformant cultures of *M. voltae* using a technique previously described (Gernhardt et al. 1990), digested with restriction enzymes, electrophoresed and transferred to nylon membranes (Roche Molecular Biochemicals) by a capillary transfer method. A digoxigenin (DIG)-labelled *flaH* DNA probe was generated by amplifying the *flaH* gene using DIG-dUTP (Roche Molecular Biochemicals) by PCR as recommended by the manufacturer. Plasmid pKJ201, which contains the *flaH* gene cloned as a *Nde*I-*Xho*I fragment, served as the template for the reaction. The primers used were overflaHfor (5′-GGAATTCCATATGAAATATGCT-AAAATAG-3′) and overflaHrev (5′-CCGCTCGAGTGCCACT-GAGGATATCTC-3′). The cycling parameters were as follows: 94°C for 5 min; 30 cycles of 94°C for 45 s, 47°C for 45 s, and 72°C for 2 min; and a final cycle with an extension time lengthened to 5 min. The PCR product (with incorporated DIG-UTP) was purified using a PCR purification kit (Qiagen). The *flaH*-specific, DIG-labelled DNA probe was denatured by boiling for 10 min and then cooled on ice prior to use in hybridizations. All Southern hybridizations were carried out at 50°C in a hybridization oven (Robbins Scientific, Sunnyvale, Calif.) with a standard hybridization buffer [$5 \times$ SSC (diluted from $20 \times$ SSC: 3 M NaCl, 0.3 M sodium citrate pH 7.0), 1% N-lauroylsarcosine, 0.2% SDS and 1% blocking reagent (Roche Molecular Biochemicals)].

Northern hybridizations were carried out using a short DNA probe generated by PCR, corresponding to the first 135 bp at the 5′ end of *flaB1*. Hybridization was carried out at 50°C with a high-SDS hybridization buffer [50% formamide, 7% SDS, $5 \times$ SSC, 50 mM Na_3PO_4 (pH 7.0), 0.1% N-lauroylsarcosine, 2% blocking reagent). High-stringency washes for both Southern and Northern hybridizations were done at 50°C as follows: 2×5 min in $2 \times$ SSC/0.1% SDS, followed by 2×15 min in $0.5 \times$ SSC/0.1% SDS. The blots were then developed using a DIG-Chemiluminescent Detection kit (Roche Molecular Biochemicals) as described by the manufacturer and then exposed to Kodak X-OMAT AR film (Mandel Scientific, Guelph, Ontario).

Immunoblotting

Whole cell and membrane fractions (Jarrell et al. 1996b) of wild-type and transformant *M. voltae* strains were subjected to SDS-PAGE (Laemmli 1970) using a minigel system and then transferred to Immobilon-P transfer membranes (Millipore, Bedford, Mass.) as previously described (Towbin et al. 1979). Polyclonal antibodies raised in chickens against each of the proteins FlaB2, FlaD, FlaE, FlaH and FlaI were produced by RCH Antibodies (Sydenham, Ontario), using C-terminally His-tagged versions of the proteins as antigens. In the case of the polyclonal anti-FlaB2 antibodies, the antibodies cross-react with both of the major flagellins in *M. voltae*, FlaB1 and FlaB2. Antibodies were used at dilutions of 1:5,000 for anti-FlaB2, anti-FlaD and anti-FlaE and at 1:1,000 for anti-FlaH and anti-FlaI. The secondary antibody was a horseradish peroxidase-conjugated rabbit anti-chicken IgY (Jackson Immunoresearch Laboratories, West Grove, Pa.) used at a 1:50,000 dilution. Blots were developed using a chemiluminescent detection kit (Roche Molecular Biochemicals) according to the manufacturer's directions.

Preflagellin peptidase assay

M. voltae FlaB2 was overexpressed in *E. coli* as previously described (Bayley and Jarrell 1999) and used as the substrate in a preflagellin peptidase assay (Correia and Jarrell 2000) under conditions [25 mM HEPES buffer containing 0.4 M KCl and 0.25% (vol/vol) Triton X-100, pH 8.5] determined to result in maximal cleavage. Peptidase-mediated cleavage of the leader peptide from the flagellin was monitored by immunoblotting analysis with polyclonal anti-FlaB2 antibodies (as described above).

Electron microscopy

Whole cells were absorbed onto copper grids and negatively stained with 2% (w/v) uranyl acetate for 1 min. Grids were viewed on a Hitachi H-7000 electron microscope operating at 75 kV.

Results

Analysis of transformants with insertions in *flaH*

Following transformation with an insertion vector containing an internal portion of *flaH*, ten cultures of streak-purified, puromycin-resistant transformants were viewed by light microscopy to evaluate motility. Eight of the ten cultures were non-motile when compared with the wild type. Electron microscopy of these non-motile cultures revealed that the cells did not have flagella (Fig. 3). Furthermore, no flagella were seen near the cells, indicating that the cultures were non-flagellated and no flagellar filaments were being made. The two puromycin-resistant cultures that were motile by light microscopy did have flagella similar to those of wild-type cells as seen by electron microscopy. Chromosomal DNA isolated from the transformed cells was digested with restriction enzymes and subjected to Southern analysis using a *flaH*-specific probe. *Ssp*I digestion of wild-type *M. voltae* DNA results in a 2.1-kb fragment that hybridizes to the probe (Fig. 4A). This fragment contains the entire *flaH* gene. *Ssp*I digestion of all the non-flagellated mutants gave a hybridization pattern that revealed a disruption in *flaH* (Fig. 4A). *Ssp*I-digested DNA from the motile transformants resulted in a 2.1-kb signal and another signal, thus demonstrating that insertion had occurred elsewhere in the chromosome and that *flaH* was not disrupted (data not shown). These motile transformants were not characterized further. Since all the *flaH*-disrupted mutants were non-flagellated and showed the same hybridization pattern on Southern analysis, a single mutant, designated H2, was chosen for further characterization.

In the case of H2, it appears that the insertion of the mutational vector into the chromosome was followed by a duplication event which generated at least two copies of the *pac* gene and reconstituted the complete internal *flaH* fragment that was cloned into the *Eco*RI site of the *pac*/pUC vector (Fig. 4B). This finding is supported by Southern blot analysis after *Eco*RI digestion of chromosomal DNA of the mutant. Three *Eco*RI fragments hybridized to the *flaH* probe (Fig. 4A), a result that can be explained by tandem insertions. In addition to the Southern data, a marker retrieval experiment was

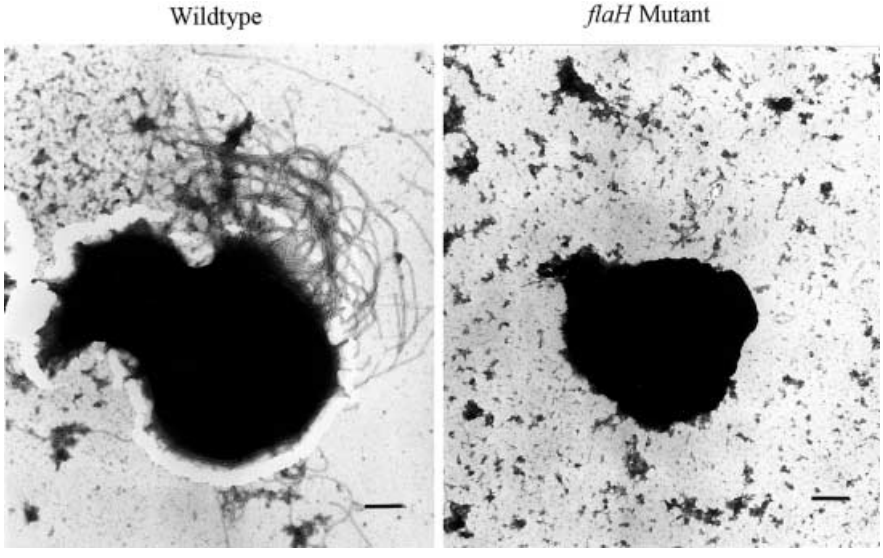

Fig. 3A, B Electron micrographs of wild-type *M. voltae* (**A**) and a *flaH* mutant (**B**). Cells were negatively stained with 2% uranyl acetate. Note the tufts of flagella extending from the cell in the wild type, and their absence in the *flaH* mutant. *Bar* 100 nm

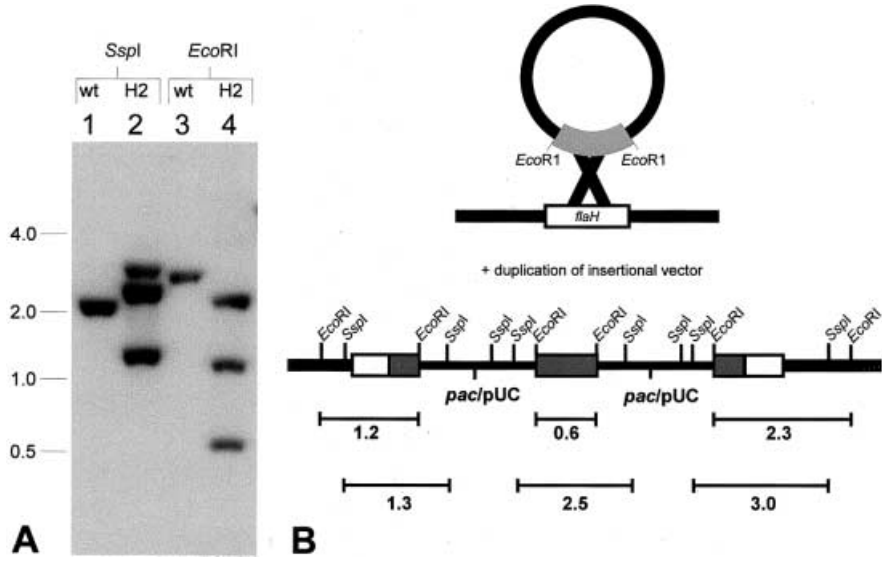

Fig. 4A, B Confirmation of insertion of the mutagenic vector into the chromosomal copy of *flaH*. **A** Southern analysis of *Ssp*I- and *Eco*RI-digested genomic DNA of wild-type *M. voltae* and a *flaH* mutant. A *flaH* probe was used to determine if *flaH* had been disrupted by the mutagenic vector. Lane 1, *Ssp*I-digested wild-type *M. voltae* genomic DNA; lane 2, *Ssp*I-digested H2 mutant genomic DNA; lane 3, *Eco*RI-digested wild-type *M. voltae* genomic DNA; lane 4, *Eco*RI-digested H2 mutant genomic DNA. **B** Schematic diagram depicting the insertion of the mutagenic plasmid into *flaH* via homologous recombination. The integration was presumably followed by a duplication event yielding two copies of the mutagenic vector in tandem (as determined by Southern hybridization analysis). The restriction map is not drawn to scale. The *thick black lines* represent the chromosome, and the *thin black lines* represent plasmid DNA that has been inserted into *flaH*. The *open boxes* represent original chromosomal *flaH* gene fragments; the *shaded boxes* represent *flaH* gene fragments derived from the insertion of the mutagenic vector. The *bars below* the restriction map correspond to the DNA fragments (sizes in kb) identified with the *flaH* probe in the Southern blot experiment shown in **A**

performed. The mutant's chromosomal DNA was digested with *Hin*dIII and then ligated at low DNA concentration to promote self-ligation. Since *Hin*dIII only cuts the *pac*/pUC vector once and there are no *Hin*dIII sites in the *flaH* sequence, when the ligated DNA is transformed into *E. coli*, only cells that have an intact replicon should survive under selection. This approach yielded transformants that harboured either one of two possible plasmids, one that had *flaH* sequence and another that had flanking DNA corresponding to that upstream of *flaH* on the chromosome. The former plasmid was subjected to DNA sequencing with the forward primer originally used to generate the internal fragment (see Materials and methods). A DNA sequence corresponding to the 560-bp *Eco*RI internal *flaH* fragment was present. This is the 560-bp fragment detected by the *flaH* probe in the Southern blot of *Eco*RI-digested mutant chromosomal DNA (Fig. 4A). Moreover, the fact that two different plasmids were obtained in the marker retrieval experiment demonstrates that tandem copies of the *pac*/pUC vector must exist in the interrupted *flaH* gene on the chromosome. The presence of tandem insertions was further shown by Southern analysis of *Kpn*I- and *Hin*dIII-digested DNA from the wild-type and the H2 mutant. Since *Kpn*I and *Hin*dIII cut only once within the vector and not at all within *flaH*, if tandem insertions had occurred, restriction enzyme digests with each of these enzymes should result in a signal corresponding to the size of the linearized plasmid in a Southern blot hybridized with a *flaH* probe. This was observed for the H2 mutant (data not shown).

Expression of putative flagella accessory genes and proteins

Northern analysis of total RNA from wild-type *M. voltae* using a *flaB1* probe results in the detection of three transcripts of 1.3, 4.2 and 5.4 kb. These mRNAs correspond to the flagellin genes and some of the putative flagella accessory genes, with the 5.4-kb message ending immediately after *flaG* (Fig. 5). Analysis of total RNA isolated from the H2 mutant revealed that all B family flagellin genes (*flaB1*, *flaB2* and *flaB3*), and at least the

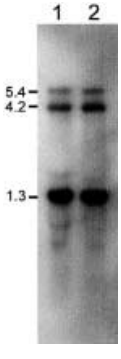

Fig. 5 Northern analysis of total RNA isolated from wild-type *M. voltae* and the H2 mutant. A short DNA probe corresponding to the first 135 bp of *flaB1* was used to detect the polycistronic transcripts. Lane 1, *M. voltae* RNA; lane 2, H2 mutant RNA

presumed flagella accessory genes *flaC* to *flaG* were transcribed, essentially at the same levels as in wild type (Fig. 5). No transcript has been detected for the *flaH* gene in wild type or in the mutants generated in this study by Northern analysis or by reverse transcriptase-PCR (data not shown), suggesting that the gene may be transcribed at a low frequency or that the message is unstable.

Experiments to determine protein levels in the H2 mutant were carried out using immunoblotting techniques. *M. voltae* P2, a non-flagellated *flaB2* insertional mutant that does not express any of the downstream genes due to a polar effect on transcription (Jarrell et al. 1996a) was included in the analyses as a negative control. Immunoblots of membrane fractions probed with polyclonal anti-FlaB2 antibodies indicated that the H2 mutant produced similar amounts of flagellin to the wild type (Fig. 6). In addition, the H2 mutant expressed the proteins FlaD and FlaE at levels similar to wild type. Immunoblotting analysis with anti-FlaH antibodies confirmed that no FlaH protein was present in the H2 mutant. Anti-FlaI antibodies demonstrated that no FlaI protein was being made in the H2 mutant, suggesting that the insertion in *flaH* may exert a polar effect on downstream transcription. FlaB1, FlaB2, FlaD, FlaE, FlaH and FlaI could not be detected in P2, the *flaB2* insertional mutant. Since no antibodies are available for FlaC, FlaF, FlaG and FlaJ, it was not possible to detect these respective proteins; however, the Northern data and the immunoblotting experiments for the H2 mutant suggest that disrupting *flaH* has no observable effect on upstream gene expression or the stability of the corresponding proteins. Cell fractionation of the H2 mutant followed by immunoblotting with anti-flagellin antibodies demonstrated that most of the flagellin was present in the membrane fraction in a processed form, similar to what is observed in wild type (data not shown). In addition, excess flagellin could not be detected in filtered culture supernatants of the H2 mutant as compared to the wild type, suggesting that the mutant is either deficient in flagellin secretion and/or assembly.

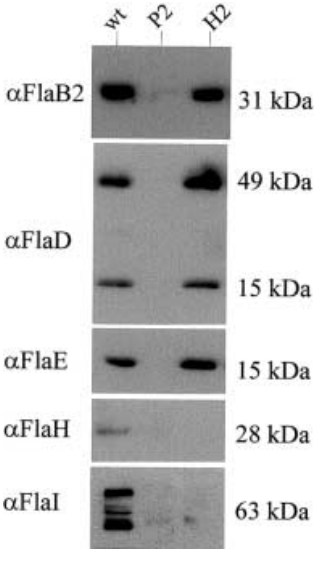

Fig. 6 Immunoblot analysis of membrane fractions of wild-type *M. voltae* (wt); a non-flagellated *M. voltae* mutant (P2) with an insertion into *flaB2* that does not express any of the known flagella accessory proteins, and the *flaH* insertion mutant (H2) generated in this study. The lane designations are valid for all the immunoblots. The polyclonal antibodies used to develop the blots are indicated to the *left* of the corresponding panel. Note that two polypeptides cross-react with the anti-FlaD polyclonal antibodies (49 and 15 kDa) in wild-type *M. voltae* but are absent in the P2 mutant that does not express FlaD. Three polypeptides cross-react with the polyclonal anti-FlaI antibodies (80, 63 and 50 kDa, which may be different forms of the same protein) in wild-type *M. voltae* but not in the P2 or H2 mutant

Assay for peptidase activity

Initial analyses of the phenotypes of flagellin gene mutants in *M. voltae* suggested that a preflagellin peptidase may be encoded by one of the downstream genes (Jarrell et al. 1996a). In order to assess whether the H2 mutant was able to cleave the leader sequence from preflagellin molecules, an in vitro preflagellin peptidase assay recently described for *M. voltae* was performed. Preflagellin peptidase activity was detected in *flaH*-disrupted mutants at a level comparable to that in wild-type cells (Fig. 7). In addition, preflagellin peptidase activity was also detected in P2, the aforementioned non-flagellated *flaB2* insertional mutant that does not express *flaC-flaG* due to a polar effect. Since it is likely that *flaHIJ* are co-transcribed, insertion of the mutagenic vector into *flaH* would inactivate all three genes. Taken together, these data demonstrate that none of the genes *flaC-flaJ* encodes the preflagellin peptidase.

Discussion

The *flaHIJ* genes in *M. voltae* were originally designated as accessory flagellar genes due to their proximity to the

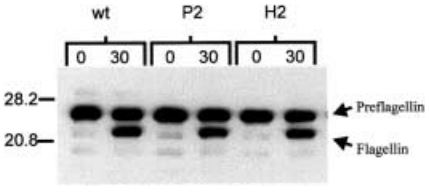

Fig. 7 Immunoblot analysis using anti-FlaB2 polyclonal antibodies to detect preflagellin peptidase activity in wild-type *M. voltae* (wt) a non-flagellated *M. voltae flaB2* mutant (P2), and the mutant generated in this study (H2). Time points (in min) refer to the time elapsed after the addition of *M. voltae* or mutant membrane preparation (which serves as the peptidase source) to *E. coli* membranes containing an overexpressed wild-type FlaB2 preflagellin in assay buffer. The reaction was stopped at the indicated time by adding electrophoresis sample buffer and then boiling prior to SDS-PAGE and immunoblotting. The *lower arrow* points to flagellin that has had its leader peptide cleaved by a membrane-located preflagellin peptidase

flagellin genes *flaB1*, *flaB2* and *flaB3* and in the belief that they are co-transcribed with the flagellin genes. However, the longest transcript initiating upstream of *flaB1* is 5.4 kb long, and would end after *flaG*. Since the distance between the end of *flaG* and the beginning of *flaH* is 138 bp, *flaHIJ* might not be transcribed with the flagellin genes but as a separate unit. Thus their involvement in flagellum biosynthesis remained unsubstantiated until the data presented in this report.

The *flaHIJ* gene cluster is likely to be co-transcribed due to the very short intergenic regions separating the genes (15 bp between *flaH* and *flaI* and 9 bp between *flaI* and *flaJ*). The insertion of the mutagenic vector into *flaH* probably causes a polar effect, due to the presence of a transcriptional terminator (Gernhardt et al. 1990), and thus also disrupts expression of *flaIJ* mRNA. Since no transcripts have been detected for *flaHIJ* in wild-type *M. voltae* or in the mutants described here, it is difficult to ascertain the expression of these genes. The finding that no FlaI could be detected in the *flaH* mutant would suggest that a polar effect was established upon insertional inactivation of *flaH*. Nonetheless, our findings demonstrate that at least one of the genes *flaHIJ* is critical for flagellum assembly in *M. voltae*.

The insertion of the *flaH* plasmid construct into the chromosomal copy of the *flaH* gene is based on a low-frequency homologous recombination event. The finding of tandem copies of the plasmid DNA in the targeted *flaH* locus was not completely unexpected. Similar cases of duplicate tandem copies of insertional vectors have been reported in *M. voltae* and its close relative *M. maripaludis* (Sandbeck and Leigh 1991; Pfeiffer et al. 1998). Transformants with tandem copies of plasmid DNA in the chromosome benefit from having a second copy of the *pac* expression cassette, which in turn confers additional resistance to puromycin on the cell. Presumably a second recombination event occurred after the insertion of the plasmid DNA resulting in a duplicate copy of the insertional plasmid arranged in tandem.

The *M. voltae* flagellins are made as preproteins that are post-translationally modified (Kalmokoff et al. 1988; Correia and Jarrell 2000). The predicted molecular masses of the FlaB1 and FlaB2 flagellins are 22.4 and 22.8 kDa, respectively; however, SDS-PAGE of purified flagellar filaments composed mainly of FlaB1 and FlaB2 results in two bands with molecular masses of 31 and 33 kDa. It is believed that some form of post-translational modification (possibly glycosylation) occurs, although none has been detected directly. In the case of the *flaH* mutant, the flagellins have the same molecular weight as those of the wild-type cells, so any putative post-translational modifications seem to be present. This suggests that none of the proteins FlaHIJ is responsible for the apparent modification of the flagellins.

Mutants for *flaA* and *flaB2* (flagellin genes) in *M. voltae* have phenotypes that suggest that the multiple flagellins present in this archaeon may have specific functions and are not interchangeable (Jarrell et al. 1996a). The *flaB2* mutant has an insertion that not only disrupts *flaB2* but also prevents expression of the downstream genes. Not too surprisingly, the protein profile of the *flaB2* mutant is different from that of the wild type, as detected by immunoblotting experiments. Immunoblotting analysis with anti-flagellin sera does not reveal the presence of the 31- and 33-kDa flagellins as seen for the wild type, but instead a novel 20-kDa protein. This cross-reactive protein was hypothesized to be an unmodified flagellin with its leader peptide intact, and suggested that one of the downstream genes encoded a preflagellin peptidase gene (Jarrell et al. 1996a). Our finding that preflagellin peptidase activity is present in both the *flaH* mutant and the *flaB2* insertional mutant indicates that none of the genes in the *M. voltae* flagellar gene family encodes the preflagellin peptidase that is responsible for cleaving the leader peptide from preflagellins.

Immunoblot analysis with antibodies against the flagellins and the putative accessory flagellar proteins FlaD and FlaE indicated that these proteins are made and are stable in the *flaH* mutant. Moreover, these proteins localized to the membrane fraction of cells, as observed in wild-type *M. voltae*. Antibodies against FlaF and FlaG are not available. Therefore, it was not possible to compare the relative levels of these proteins in the *flaH* mutant with those in wild-type cells, although considering the transcript lengths from the Northern experiments it is expected that similar levels of these proteins would occur in both types of cells. Analysis of culture supernatants and subcellular fractions of the *flaH* mutant did not reveal any significant differences in flagellin localization compared to the wild-type cells. It appears that the *flaH* mutant can make flagellin, and target it to the membrane; however, the flagellin does not leave the membrane and is not assembled into a flagellum. In addition, no obvious structural intermediates or detached flagella could be found by electron microscopy. The *flaH* mutant appears to be capable of expressing the flagellin genes and *flaC-G* at levels equivalent to those in the wild type. This is suggested by the Northern data and by immunoblot analysis with the antibodies available. These observations, as well as protein localization data, implicate the *flaHIJ* genes and possibly other genes in secretion or assembly of flagellar components. Thus in *M. voltae*, the flagellins and accessory proteins FlaC-G are not sufficient for the assembly of a flagellum.

It is been demonstrated that FlaH and FlaI locate to the membrane fraction of cells (Thomas and Jarrell, unpublished data). Although no localization data are available for FlaJ, it is believed to be a membrane protein, since structure prediction algorithms identify 7–9 transmembrane domains. Both FlaH and FlaI have nucleotide binding domains that are probably located in a cytoplasmic loop in each of the proteins. It is hypothesized that these proteins may provide the energy that is presumably required for the secretion of archaeal flagellar proteins. FlaI does share homology with PilT (and its homologues), a protein demonstrated to be

involved the type IV pilus function in *Pseudomonas aeruginosa* (Whitchurch et al. 1990). Archaeal flagellins also share N-terminal sequence homology with type IV pilins (Faguy et al. 1994). A terminal branch of the type II secretion system is responsible for the assembly of type IV pili in a number of pathogenic bacteria (Strom and Lory 1993). It is believed that archaeal flagellins are secreted using a system similar to the type II secretion systems in bacteria. Analysis of complete genome sequences of archaeal species has identified type II secretion system homologues but only one gene with homology to the type IV pilus system (*flaI*). Further experiments to identify archaeal proteins involved in protein secretion are needed to elucidate flagellar biosynthesis.

Prior to this study, there were no biochemical or genetic data characterizing the putative flagella accessory genes *flaC-flaJ* in any archaeal species. The finding that disruption of *flaH* (and probably *flaI* and *flaJ* due to polar effects) results in a non-flagellated phenotype for the first time directly implicates this gene family in flagellum biosynthesis or assembly. Furthermore, the findings presented here suggest that other genes outside of the known *M. voltae* flagella gene family are involved in flagellar secretion and assembly. For example, the gene coding for preflagellin peptidase activity is not yet known. We are currently generating mutants using insertional activation to identify other genes involved in flagellar morphogenesis.

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