Posttranslational Processing of *Methanococcus voltae* Preflagellin by Preflagellin Peptidases of *M. voltae* and Other Methanogens

by

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Abstract

Methanococcus voltae is a mesophilic archaeon with flagella composed of flagellins that are initially made as preflagellins, with 11 to 12 amino acid leader peptides that are cleaved prior to incorporation into the growing filament. The enzymatic activity responsible for cleavage of the leader peptide was demonstrated in a system based on an *in vitro* type IV prepilin peptidase assay. Preflagellin substrate for use in the detection of preflagellin-specific peptidase activity was prepared by overexpression of *M. voltae* FlaB1 or FlaB2 preflagellin in *Escherichia coli* and combined *in vitro* with methanogen membrane (as enzyme source). Peptidase activity was demonstrated in immunoblotting experiments using anti-flagellin antibody to detect unprocessed and processed flagellin subunits. Correct processing of the preflagellin to the mature flagellin was shown directly by comparison of the N-terminal sequences of the two flagellin species. For the conditions tested, *M. voltae* FlaB2 preflagellin peptidase activity was maximal near 40 °C pH 8.5 and in the presence of 0.4 M KCl with 0.25% (vol/vol) Triton X-100 and did not require the addition of acidic phospholipids (which is an essential component of the prepilin assay). Preflagellin peptidase activity was observed in the membranes of several flagellated methanogens, including mesophiles (*M. voltae*, *Methanococcus vannielii*, *Methanococcus maripaludis* and *Methanococcus deltae*), a thermophile (*Methanococcus thermolithotrophicus*) and a hyperthermophile (*Methanococcus jannaschii*). No preflagellin peptidase activity was observed in the membranes of *Methanococcus igneus*, *Methanogenium cariaci* or *Methanoculleus marisnigri*. This contribution represents the first report of a preflagellin peptidase.

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List of Abbreviations

A	ampere
Ado-Met	*S*-adenosyl-L-methionine
bp	base pair(s)
DIG	Digoxigenin
EDTA	ethylenediamine tetraacetic acid
ESB	electrophoresis sample buffer
FD	Faraday
g	gram(s)
h	hour(s)
IPTG	isopropylthio-β-galactosidase
kb	kilobase
kDa	kiloDalton
L	litre(s)
M	molar
MCS	multiple cloning site
min	minute(s)
M_r	relative mobility
MTase	methyl-transferase
O.D.	optical density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
rpm	rotation per minute
RT-PCR	reverse transcriptase PCR
SDS	Sodium Dodecyl Sulfate
sec	second(s)
SSC	standard saline citrate
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine

Tris	Tris (hydroxymethyl) aminomethane
V	volt(s)
vol	volume
wt	weight
X-gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside
Ω	Ohm(s)

Chapter 1. Introduction

The domain Archaea

The urkingdoms, eubacteria, urkaryotes and archaebacteria were first proposed by Woese and Fox in the 1970s based mainly on comparisons of small subunit (SSU) rRNA sequence data (Woese and Fox 1977), and later renamed the Bacteria, the Eucarya and the Archaea (Woese et al. 1990). The Archaea are a group of phylogenetically distinct microorganisms, divided into two kingdoms (Figure 1), the Euryarchaeota (comprising methanogens, extreme halophiles and some hyperthermophiles) and the Crenarchaeota (initially thought to include only hyperthermophiles, but now known to include many, as yet uncultured, non-thermophilic species [Pace 1997]).

Originally, the Domain Archaea was thought to consist mainly of organisms found in unusual or extreme environments (Table 1), such as extreme halophiles, which are able to grow in saturating concentrations of NaCl; the methanogens, which require extremely anaerobic conditions for growth and methane production; and the sulfur-dependent thermophiles, which are capable of growth at high temperatures and, often, highly acidic conditions (Jarrell et al. 1999). However, low-temperature crenarchaeota have recently been discovered that grow under the less extreme conditions favored by most bacteria, including mesophilic (temperatures usually between 20 and 45°C) and even psychrophilic (temperatures usually below 15°C) environments. The sources of these microbes include freshwater sediments (MacGregor et al. 1997; Schleper et al. 1997) and subsurface ocean waters (Fuhrman et al. 1993; DeLong 1992). These findings support the notion that, although often found in extreme environments, the Archaea are truly ubiquitous and may be present in habitats previously thought to be too "normal."

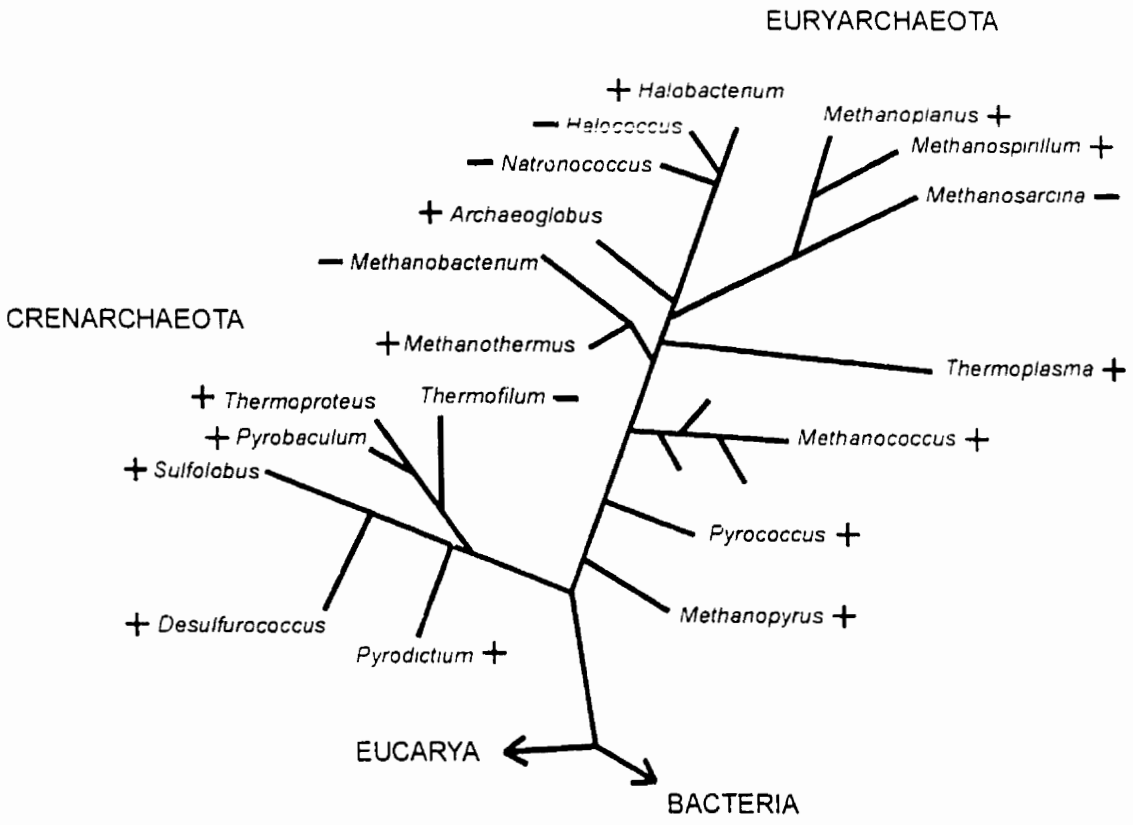


Figure 1. Phylogenetic tree of the Archaea demonstrating the ubiquitous nature of flagellation throughout this domain, including members of the Crenarchaeota and Euryarchaeota. +, flagellated, -, not flagellated. Updated from Jarrell et al. (1996b).

Table 1. Environmental extremes inhabited by archaea.

Extreme feature	Natural habitat	Examples
Temperature	Deep-sea hydrothermal vents, Hot springs, Volcanic areas	*Pyrolobus fumarii* (growth to 113°C)
pH	Solfataric springs	*Picrophilus oshimae* (growth to near pH 0)
Salt	Dead Sea, Great Salt Lake, salterns	*Halobacterium salinarum* (growth in 5.2 M NaCl)
Anaerobiosis	Rumen, sewage digesters, swamps	*Methanospirillum hungatei* (redox potential of less than −330 mV)
Salt-pH	Soda lakes	*Natronobacterium magadii* (pH 8.5 to 11, 2 to 5.2 M NaCl)
Temperature-pH	Acidic hot springs, coal refuse piles	*Sulfolobus acidocaldarius* (pH 2, 75°C)
Anaerobiosis-Salt	Great Salt Lake sediment, saline cyanobacterial mats	*Methanohalophilus mahii* (anaerobic, 2 M NaCl)

This vast array of environments in which archaea flourish, from one extreme to the other, have intrigued scientists for the past 20 years. In this time, the known members of the Archaea have been greatly expanded, mainly by the isolation of organisms that extend the limits of life on both the temperature and pH scales.

Thermophilic archaea grow optimally at temperatures between 50°C and 70°C. They are predominantly sulfur oxidizing, can be aerobes or anaerobes and occur commonly in hot springs, terrestrial solfataric fields, marine volcanic vents and in deep-sea hydrothermal vents (Aravalli et al. 1998). Representative species include *Methanococcus thermolithotrophicus*, which grows between 30 and 70°C with an optimum at 65°C (Huber et al. 1982) and *Thermoplasma acidophilum*, which grows optimally at 60°C and pH 1.8 to 2.0. Interestingly, *Thermoplasma* spp. lack a cell wall. The glycan chains of their cytoplasmic membranes are directed towards the outside of the cells, forming a glycocalyx, which may partly function as a cell wall (König 1988). Hyperthermophilic archaea grow optimally at temperatures >80°C. In fact, *Pyrolobus fumarii* grows optimally at 106°C, but can grow at 113°C and even survive 1 hour of autoclaving at 121°C (Blöchl et al. 1997). By contrast, the most thermophilic bacteria are members of the genera *Thermotoga* and *Aquifex*, which grow optimally at approximately 85°C.

Methanogens are obligate anaerobes that generate energy from methanogenesis, the production of methane from substrates including CO_2/H_2 and acetate. Representative genera include *Methanobacterium*, *Methanococcus*, *Methanogenium* and *Methanoculleus*. They are ubiquitous organisms found in a variety of environments rich in organic matter, including aquatic sediments, the rumen of animals, sewage sludge

digesters, human oral cavities and at deep-sea geothermal vents. Methanogens are often of ecological significance (e.g., anaerobic digesters use methanogens to degrade sewage sludge to H_2, CO_2 and acetate). Some of the best-studied methanogens are members of the genus *Methanococcus*.

Genus *Methanococcus*

The family *Methanococcaceae* includes one genus, *Methanococcus*. As their name implies, members of the genus are cocci, usually 1 to 2 μm in diameter. These obligately anaerobic methane-producing archaea have been isolated from salt marsh, marine and estuarine sediments. They are motile by means of tufts of flagella and species grow optimally at temperatures ranging from mesophilic to extremely thermophilic. Table 2 summarises some general characteristics of the different *Methanococcus* spp., including mesophiles (*Methanococcus voltae*, *Methanococcus deltae*, *Methanococcus vannielii* and *Methanococcus deltae*), a thermophile (*Methanococcus thermolithotrophicus*) and hyperthermophiles (*Methanococcus jannaschii* and *Methanococcus igneus*).

Perhaps the best-studied organism of the *Methanococcus* genus is *M. voltae*, a mesophilic, marine coccus. The genome of this methanogenic archaeon consists of one circular double-stranded DNA molecule of approximately 1900 kb (Sitzmann and Klein 1991). Cell envelopes consist of a hexagonally arranged S layer external to the plasma membrane. These S layer protein subunits have a molecular weight of approximately 76,000 (Jarrell and Koval 1989). The insertion of a flagellum into this unusual type of envelope, characteristic of many archaea, is expected to be different from that observed

Table 2. Some characteristic properties of species of the genus *Methanococcus*

Species	Source	Cell diameter (μm)	Temperature optimum (°C)	pH optimum	Optimal salinity (M of NaCl)	Required growth factors[a]	G + C content (mol%)
M. voltae	Estuary sediment	1.5	35-40	6.5-8.0	0.2-0.4	Ac, ile, leu	29-32
M. vannielii	Marine mud	1.3	35-40	7.0-9.0	0.1-0.4	None	33
M. deltae	River sediment	1.0-1.5	37	n.d.[b]	0.6-0.68	None	40.5
M. maripaludis	Salt marsh sediment	1.0	35-40	6.5-8.0	0.2-0.4	None	33-34
M. thermolithotrophicus	Heated sea sediment	1.0	65	7.0	0.3-0.7	None	34
M. jannaschii	Hydrothermal vent	1.0	85	6.0-7.0	0.4-0.7	None	31-33
M. igneus[c]	Hydrothermal vent	1.3-1.8	88	5.7	0.2	None	31

[a]None, autotrophic growth with CO_2 as the sole carbon source, Ac, acetate, ile, isoleucine, leu, leucine.
[b]n.d., Not determined.
[c]It is unclear whether the few filamentous structures observed on *M. igneus* are in fact flagella.

in the multilayered envelopes of bacteria. In *M. voltae*, a polar membrane-like structure is usually found associated with the flagellar insertion point. These structures consist of a bar-like array of stalks and knobs arising from the inner surface of the plasma membrane and extending into the cytoplasm approximately 20 nm (Koval and Jarrell 1987).

The archaeal flagellum

All of the major subgroupings of archaea, including methanogens, extreme halophiles, and sulfur-dependent thermophiles and hyperthermophiles, have members that possess flagella (Figure 1 [p. 2]) that look superficially like bacterial flagella (Jarrell et al. 1996b). Surprisingly, even members of the genus *Thermoplasma*, a thermoacidophile that lacks a cell wall, are flagellated. However, recent evidence has indicated that the archaeal flagellum is a unique motility structure, distinct from that of bacteria in composition and likely assembly (Bayley and Jarrell 1998; Jarrell et al. 1996b). Four pieces of evidence support this hypothesis. First, there is no sequence similarity between archaeal flagellins and bacterial flagellins. Surprisingly, archaeal flagellins instead share sequence similarity and a hydrophobic N-terminal region with type IV pilins (Faguy et al. 1994a), which includes pilins from *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Neisseria meningiditis*, *Moraxella bovis*, *Dichelobacter* (formerly *Bacteroides*) *nosodus* and *Vibrio cholerae*. The pilin subunits of this family are synthesized as secretory precursors (prepilins) with short, basic, N-terminal leader peptides (Strom and Lory 1993). Furthermore, one gene, *flaI*, located immediately downstream of the flagellins in *M. voltae* and possibly cotranscribed with the flagellin genes, encodes a protein homologous to PilT, a nucleotide binding protein of type IV

pilus gene families (Bayley and Jarrell 1998). Second, archaeal flagellins are often glycosylated (Table 3). In halobacteria this modification has been shown to occur outside the cytoplasmic membrane (Sumper 1987), a location difficult to reconcile with the bacterial mode of flagella assembly where the flagellins pass through the hollow filament and emerge for incorporation at the distal tip (Jones and Aizawa 1991). The thinner diameter of the archaeal flagellar filament (10 to 15 nm [Koval and Jarrell 1987]) compared to the bacterial structure (approximately 20 nm [Macnab and DeRosier 1988]) may preclude passage of the flagellins through a central core. Rather, the archaeal flagellum may be assembled in a manner more similar to that of type I pili (in which new pilins are added at the base [Lowe et al. 1987]), which, although not demonstrated, is likely the basic mechanism in the assembly of type IV pili (Mattick and Alm 1995). Third, analyses of the completely sequenced genomes of the flagellated archaea have failed to reveal genes similar to any of the genes that encode structural proteins of the bacterial flagellum, indicating that the structural components of the archaeal flagellum are distinct (Faguy and Jarrell 1999). Finally, the assembly of archaeal flagella requires the posttranslational cleavage of a short (11 or 12 amino acids) leader peptide (Bayley and Jarrell 1998; Kalmokoff and Jarrell 1991) from the precursor form of the flagellin monomer (preflagellin) before its incorporation into the growing filament. Bacterial flagellins are not made with leader peptides (Jones and Aizawa 1991). It should be noted that Brahamsha and Greenberg (1989) reported the identification of a 37.5 kDa "flagellin" in *Spirochaeta aurantia* with a putative signal sequence and signal peptide cleavage site, however, this "flagellin" shows no homology to other bacterial flagellins (Brahamsha and Greenberg 1989). Instead, this molecule represents a flagellar sheath

Table 3. Characteristics of archaeal flagellins.

Organism	No. of flagellins[a]	Flagellin Size (kDa)[a]	Glycosylation[b]	No. of flagellin genes	Predicted molecular weight (kDa) of flagellins from gene sequence
Aeropyrum pernix	n.d.[c]	n.d.	n.d.	2	21.4, 25.9
Archaeoglobus fulgidus	n.d.	n.d.	n.d.	2	21.8, 22.3
Halobacterium saccharovorum	3	40, 47, 50	+	nd	n.d.
Halobacterium salinarum R_1M_1	3	36, 30, 26	+	5 or more	20.5, 20.5, 20.6, 20.6, 20.7
Halobacterium salinarum RCM1769	4	25, 27, 29, 30	+	n.d.	n.d.
Halobacterium volcanii	5	26,29, 32, 39, 43	+	n.d.	n.d.
Methanococcus deltae	2	27, 32	+	n.d.	n.d.
Methanococcus jannaschii	2	27, 32	-	3	22.5, 22.7, 23.1
Methanococcus maripaludis	3 or 4	27.5 to 33	n.d.	3 or more	n.d.
Methanococcus thermolithotrophicus	3	27.5 to 33	-	3 or more	n.d.
Methanococcus vannielii	2	28.6, 30.8	-	3 or more	21.8, 22.5, 23.1
Methanococcus voltae	2	31, 33	-	4 or more	22.5, 22.8, 23.9, 25.5
Methanoculleus marisnigri	2	25.5, 31	-	n.d	n.d.
Methanospirillum hungatei GP1	2	24, 25	+	n.d.	n.d.
Methanospirillum hungatei JF1	2	24, 25, 35	+	n.d.	n.d.
Methanothermus fervidus	3	24, 25, 34	+	n.d.	n.d.
Natronobacterium magadii	4	45, 59, 60, 105	+	n.d.	n.d.
Natronobacterium pharaonis 12	2	41, 84	+	n.d.	n.d.
Natronobacterium pharaonis (DSM 2160)	4	80, 84, 86, 88	-	n.d.	n.d.
Pyrococcus furiosus	2	32, 32.5	+	n.d.	n.d.
Pyrococcus horikoshii	n.d.	n.d.	n.d.	2	21.8, 22.3
Sulfolobus shibatae	2	31, 33	+	n.d	n.d.
Thermococcus stetteri	2	27, 28	+	n.d	n.d.
Thermoplasma acidophilum	1	38	+	n.d.	n.d.
Thermoplasma volcanium	1	41	+	n.d	n.d.

[a]As determined by SDS-PAGE of purified flagella filaments

[b]As determined by thymol-sulfuric acid or periodic acid-Schiff staining

[c]n.d., Not determined.

protein and not a flagellin. Together these data suggest that archaeal flagella may be assembled in a manner more similar to type IV pili than to bacterial flagella.

Physical characteristics of the archaeal flagellum

Purified archaeal flagellar filaments are generally 10 to 15 nm in diameter (Koval and Jarrell 1987) and composed of multiple flagellins rather than a single flagellin species common to most bacterial flagella, although a few bacterial species are composed of multiple flagellins (Wilson and Beveridge 1993; Jones and Aizawa 1991; Macnab 1990). Much thinner filaments (3 to 5 nm in diameter) have been observed in flagellar preparations of the halophile *Natronobacterium magadii* incubated under decreasing NaCl concentrations (Polosina et al. 1998). These thin filaments have been termed protofilaments and it has been suggested that these are intermediates in the dissociation of flagellar filaments. Similar thin filaments have also been observed upon long-term storage of other archaeal flagella preparations, such as those from *Thermoplasma* spp (Faguy et al. 1996).

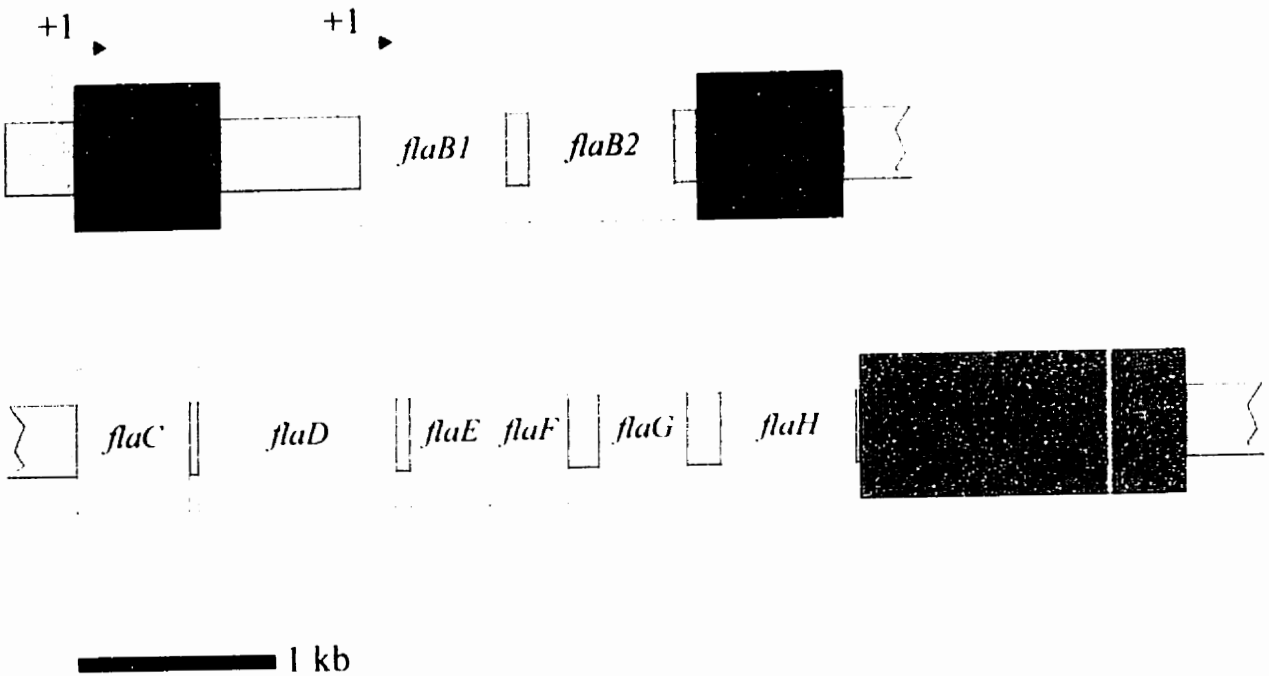
The structure of archaeal flagellar filaments composed of glycosylated flagellins has been shown to be sensitive to treatment with Triton X-100, a detergent commonly used for the isolation of basal-body-hook-filament complexes from bacteria. However, even electron microscopy of detergent–resistant flagella filaments has yet to reveal any ring-like or other basal body substructures characteristic of bacterial flagella. Instead, knob-shaped structures have been identified on flagella isolated from methanogens and other archaea by phase separation with Triton X-114 (Kalmokoff et al. 1988) or detergent treatment of envelope fragments (Faguy et al. 1994b). These structures possibly only

represent the portion of the basal structure resistant to detergent treatment. In *Halobacterium salinarum* (formerly *H. halobium*), the flagellar bundle is inserted into a differentiated polar cap structure (Kupper et al. 1994), which may be analagous to the polar membrane-like structure described previously for *M. voltae* (Koval and Jarrell 1987).

Archaeal flagellin gene families

Previously, in *M. voltae*, 4 flagellin genes (*flaA*, *flaB1*, *flaB2* and *flaB3*) encoded by two transcriptional units (Figure 2) were identified (Kalmokoff and Jarrell 1991). One transcriptional unit contains only *flaA*; the other, polycistronic transcriptional unit (of at least 5.4 kb in length), containing *flaB1*, *flaB2*, *flaB3* and a number of presumed flagella accessory genes, initiates at *flaB1* and extends to at least the end of *flaG* (Bayley et al. 1999; Kalmokoff and Jarrell 1991). Transcription of *flaA* appears to be low and the mRNA transcript coding for the gene products downstream of FlaB2 appears to be short-lived. As well, the rare codon usage in FlaB3 would also indicate it is produced in much lower amounts (Kalmokoff and Jarrell 1991). It is possible that the mRNA may extend even further beyond *flaG*, to include more of the downstream genes, however, this has yet to be shown. Gene families homologous to the *M. voltae* flagellar operon have been identified upon investigation of *H. salinarum* (Gerl and Sumper 1988), *M. maripaludis*, *M. thermolithotrophicus* (Thomas and Jarrell 1999), the complete genome sequences of the flagellated archaea *Archaeoglobus fulgidus* (Klenk et al. 1997), *M. jannaschii* (Bult et al. 1996), *Pyrococcus horikoshii* (Kawarabayasi et al. 1998) and *Aeropyrum pernix* (Kawarabayasi et al. 1999), but not in the nonflagellated methanogen,

Figure 2. Schematic of the *M. voltae* flagellin gene region. The flagellin genes *flaA*, *flaB1*, *flaB2* and *flaB3* are based on Kalmokoff and Jarrell (1991). Seven additional genes were identified by Bayley and Jarrell (unpublished data) and were labeled *flaC* through *flaI*. Relative transcriptional start sites are indicated by "+1." The product of the *flaI* gene (orange) is homologous to PilT of the type IV pilus gene families.


+1
+1
flaB1
flaB2
flaC
flaD
flaE
flaF
flaG
flaH
1 kb

Methanobacterium thermoautotrophicum (Smith et al. 1997). Characteristics of the flagellins and flagellin genes of archaea are given in Table 3. None of the archaeal sequences described to date have any significant sequence similarity to bacterial flagellins. However, all of the archaeal flagellins have extensive N-terminal homology among themselves (Kalmokoff et al. 1990).

N-terminal (Kalmokoff et al. 1990), transcriptional (Kalmokoff and Jarrell 1991) and mutational (Jarrell et al. 1996a) analyses have provided evidence suggesting that *flaB1* and *flaB2* code for the major flagellins in *M. voltae*. Isolated *M. voltae* flagellar filaments are composed of two flagellins of molecular weights 31,000 and 33,000 (Kalmokoff et al. 1988). The amino acid sequence for each of the cloned flagellins predicted from the gene sequences (Kalmokoff and Jarrell 1991) contains an additional 11 or 12 amino acids not present on the N terminus of the 31,000-M_r flagellin, which confirms that this sequence represents a leader peptide. Similarly, in the related methanogen, *M. vannielii*, comparison of the N-terminal sequences obtained from the two major flagellins of purified flagellar filaments with the deduced amino acid sequence of the cloned genes definitively identified the presence of 12 amino acid leader peptides on the flagellins FlaB1 and FlaB2 (Bayley et al. 1998). The presence of leader peptides on archaeal flagellins indicated that an enzymatic activity must be present in archaeal cells to process the preflagellins. Past work (Jarrell et al. 1996a) with a non-flagellated *M. voltae* mutant carrying a vector insertion in *flaB2* (which has a polar effect on the co-transcribed downstream genes) has suggested that a preflagellin peptidase may be encoded within the polycistronic transcriptional unit downstream of the *flaB* flagellin genes.

Examination of the entire flagella gene region, including flagellin and putative flagella accessory genes in *M. voltae* and the completely sequenced archaeal genomes reveal that the region extends approximately 7.5 to 14 kb, depending on the archaeon. Flagellin gene clusters vary with respect to composition and number of genes, suggesting that some may not be essential or have additional functions. A number of genes are common to all families: *flaHIJ*. Interestingly, *A. fulgidus* lacks the equivalents of *flaCDEFG* common to *M. voltae*, *M. jannaschii*, *P. horikoshii* and *P. abyssi* (N.B., *P. abyssi* lacks a *flaF* equivalent). Aside from FlaI, which is homologous to the nucleotide binding protein PilT, the other archaeal proteins encoded by the flagellin gene families do not show any homology to any other proteins in GenBank. These genes likely encode proteins unique to flagellar synthesis, assembly and function in the Archaea.

Archaeal flagellin primary sequence analysis

N-terminal sequences of flagellins have been obtained from a variety of archaea, either by direct protein sequencing of purified protein or deduced from the gene sequence obtained from cloned flagellin genes or from genome sequencing projects. Over thirty N-terminal sequences of archaeal flagellins from at least fourteen different organisms are now available (Kawarabayasi et al. 1999; Kawarabayasi et al. 1998; Klenk et al. 1997; Jarrell et al. 1996b; Bult et al. 1996). These include flagellins from members of both archaeal kingdoms (the Crenarchaeota and the Euryarchaeota) and include mesophiles, thermophiles and hyperthermophiles. These N-termini are conserved and very hydrophobic in relation to the remainder of the molecule (Kalmokoff and Jarrell 1991; Kalmokoff et al. 1990). This region may be analogous to the conserved N-termini of

bacterial type IV pilins. In both cases, this region may play an important role in the assembly and/or function of the pilus or flagellum, respectively. In addition, this highly conserved region may play an essential role in the recognition of preflagellin by the preflagellin peptidase. It is known in the prepilin system that the +5 glutamic acid is needed for proper methylation of the N-terminal phenylalanine residue by the prepilin peptidase (MacDonald et al. 1993; Strom and Lory 1991).

Although glycosylation of bacterial flagellins is extremely rare, many archaeal flagellins are apparently glycosylated (Kalmokoff et al. 1992). Glycosylation of *H. salinarum* flagellins have been studied extensively. Halobacterial flagella consist of three related glycoproteins. N-linked glycosylation occurs at typical Asn-X-Thr/Ser glycosylation sites (Lechner and Weiland 1989). The precise function of the glycosylation and the effect it might have on the properties of the filaments is unknown at present. However, it was observed that treatment of *M. deltae* with the glycosylation-inhibiting antibiotic bacitracin results in the disappearance of normal flagella (~ 12 nm in diameter). Instead, thinner appendages (~ 7 nm in diamter) are produced (Bayley et al. 1993). Apparently, a minimum amount of glycosylation is essential for normal flagellum assembly. *M. deltae* is the only member of the Methanococcales for which glycosylated flagellins have been demonstrated (Bayley et al. 1993).

Bacterial type IV pili

Several bacterial species display surface appendages termed pili (or fimbriae). These pili are essential to a variety of functions, including adhesion to host cell surfaces, twitching motility, modulation of target cell specificity and bacteriophage adsorption

(Soto and Hultgren 1999). Distinct families of pili have been identified based on sequence similarities of their major subunits, pilins (Strom and Lory 1993), as well as the conservation of their assembly and regulatory components (Lory and Strom 1997).

The type IV pili family includes a heterogeneous group of taxonomically unrelated Gram-negative organisms, whose pilins may be further divided into 2 groups. Group A includes pilins from *P. aeruginosa*, pathogenic *Neisseria* (e.g., *N. gonorrhoeae*, *N. meningitidis*), *Moraxella bovis*, *D. nodosus* and *V. cholerae* and enteropathogenic *E. coli* (EPEC) (Strom and Lory 1993). More recently, this class was expanded to include the pili of *Moraxella lacunata*, *Moraxella nonliquefaciens*, *Branhamella catarrhalis*, *Eikenella corrodens*, *Aeromonas hydrophila* and *Myxococcus xanthus* (Alm and Mattick 1997). The pilin subunits of this group are synthesized as secretory precursors (prepilins) with short (6 or 7 amino acids), basic, N-terminal leader peptides. These leader peptides are removed by endoproteolyic cleavage between an invariant glycine and phenylalanine residue prior to incorporation of the pilin monomers into fimbriae. The amino acid sequence homology is most evident near the amino terminus of the mature pilin. This domain is characterized by strong hydrophobic amino acids extending thirty to thirty-five amino acid positions from the mature amino terminus. The central segment of the pilin monomers are notably less homologous and contain the variable domains that constitute the antigenic epitopes of these pili. Finally, a second region of homology is located near the carboxy terminus. This domain contains a characteristic pair of cysteines responsible for the formation of a disulfide loop (Strom and Lory 1993).

The type IV pilin family group B includes subunits of the toxin-coregulated pili (Tcp) of *V. cholerae*, bundle-forming pili (Bfp) of enteropathogenic *Escherichia coli*

(EPEC) (Strom and Lory 1993) and Longus pili of enterotoxigenic *E. coli* (ETEC) (Girón et al. 1997). The TcpA (the Tcp subunit) and BfpA (the BFP subunit) precursors are fashioned with longer leader peptides than those of group A pilins: 25 and 13 amino acids, respectively. Furthermore, the first amino acid of mature TcpA and BfpA are methylated methionine and methylated lecuine, respectively, as opposed to the invariant methylated phenylalanine of group A pilins.

Type IV pilus biogenesis involves the synthesis of major and minor subunits, posttranslational modifications of these subunits and assembly into a functional organelle. This process likely requires one or more accessory proteins, including the following: (1) a prepilin peptidase responsible for the posttranslational cleavage of the prepilin N-terminal leader peptide and *N*-methylation of the resulting N-terminal amino acid; (2) an integral cytoplasmic membrane protein that may serve as a scaffold for pilus assembly; (3) a hydrophilic nucleotide-binding protein involved in the translocation of macromolecules across the cytoplasmic membrane; and (4) an outer membrane component responsible for translocation of the assembled pilus across the outer membrane (Strom and Lory 1993).

P. aeruginosa PilD

The prepilin peptidases of various microorganisms range from 248 to 290 amino acids and have similar predicted secondary structures based on various computer analyses of the protein sequences, including a strong hydrophobic character signified by 5 to 8 stretches of hydrophobic amino acids (Lory 1994). This suggested that the enzyme is very likely an integral membrane protein, which was confirmed by the identification of

prepilin peptidase activity in the cytoplasmic membrane fraction of *P. aeruginosa* (Strom et al. 1994). In *P. aeruginosa*, the prepilin peptidase is encoded by the *pilD* gene (Nunn and Lory 1991) and is a bifunctional enzyme responsible for cleavage of the prepilin leader peptide and methylation of the resulting N-terminal phenylalanine residue. The predicted amino acid sequence of the *P. aeruginosa* prepilin peptidase, PilD, revealed a hydrophobic protein with 5 to 6 transmembrane spanning helices and a large, hydrophilic N-terminal domain. β-lactamase and alkaline phosphatase/β-galactosidase fusions confirmed the cytoplasmic location of the most conserved N-terminal region (aa^{40} to aa^{110}), which contains a cluster of cysteine residues implicated in the catalytic activities of the peptidase. This was confirmed by inhibition of leader peptidase and methyl-transferase (MTase) activities with thiol-specific reagents, including *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), *p*-chloromercuriphenylsulfonate (PCMPS) and iodoacetamide (IOA) (Lory 1994). Four of only 5 cysteine residues of *P. aeruginosa* PilD are found in this highly conserved cytoplasmic domain (at positions 72, 75 and 97, 100). These cysteine residues are arranged in a two-pair motif (each residue is generally separated by two amino acids), with each pair separated by twenty-one largely hydrophilic amino acids (C-X-X-C $...X_{21}...$ C-X-X-C). This region is conserved among the PilD homologues, TcpJ of *V. cholerae*, PulO of *Klebsiella oxytoca*, ComC of *Bacillus subtilis* and PilD of *N. gonorrhoeae*. Interestingly, two PilD homologues, HopD of *Haemophilus influenzae* and XpsO of *Xanthomonas campestris*, are completely devoid of cysteine residues in the conserved region while still retaining homology for this N-terminal domain. In fact, the cloned *xpsO* gene complements a *pilD* mutation in *P. aeruginosa* (Lory and Strom 1997). Single substitutions of these four cysteine residues

with glycine or serine resulted in substantially reduced leader peptidase and MTase activities. Therefore, the active sites of the enzyme are within domains of the cytoplasmic segment of PilD. These cysteine residues may contribute to an overall confirmation of PilD that is essential for effective catalysis of its substrates. Similar substitutions of the single, and most N-terminal, cysteine residue (aa^{17}) had no effect on enzymatic activity (Strom and Lory 1993).

The concurrent loss of both leader peptidase and MTase activities would support the idea that both active sites are closely linked. However, the methylation of pre-cleaved substrate demonstrates that the substrate for methylation does not have to be generated by the same enzyme. Furthermore, MTase inhibition with sinefungin, a structural analogue of the methyl donor *S*-adenosyl-L-methionine (Ado-Met) had no effect on leader peptidase activity (Lory and Strom 1997). These observations confirm that both reactions involve separate active sites in distinct domains of the PilD molecule.

Prepilin leader peptides

The prepilin leader peptide may serve several possible functions. It may communicate information regarding its destination in one or more early steps in the assembly process. The leader peptide may prevent premature polymerization of the subunit monomers before translocation across the cytoplasmic membrane. It may be required for conformational maintenance of the prepilin during biogenesis. Finally, the leader peptide may protect the prepilins from proteolytic degradation (Lory 1994).

Although all type IV prepilins contain reasonable signal peptidase cleavage sites, they are not cleaved by signal peptidase I. Rather, the prepilin peptidase performs a

function similar to that of signal peptidase I, cleaving the signal peptides and releasing the processed subunits into the periplasm (Lory 1994). The N-terminal hydrophobic amino acids of *N. gonorrhoeae* prepilin serve as a trans-membrane signal for the translocation of prepilin across the inner membrane via the general secretory pathway (Fussenegger et al. 1997). In the model of *N. gonorrhoeae* type IV pilus biogenesis (Fussenegger et al. 1997), the prepilins remain attached to the cytoplasmic membrane via their hydrophobic N-termini (with their hydrophilic heads directed towards the periplasm and their leader peptides exposed to the cytoplasm). These embedded subunits then locally associate with an inner membrane assembly complex including the prepilin peptidase. Cleavage of the prepilin leader may then facilitate the release of mature pilin and its incorporation into the fimbrial structure. The role of N-methylation on the other hand is unclear. N-methylation may serve to protect against degradation by amino peptidases or to facilitate interaction with potential chaperones, however, these hypotheses have yet to be examined (Lory 1994)

Complete prepilin cleavage by the prepilin peptidase absolutely requires glycine at position −1, at least in *P. aeruginosa*, and a hydrophobic amino acid, frequently phenylalanine (70% of potential substrates), at position +1. The N-terminal amino acid of the mature pilin is conserved among group A pilins. Methionine (>10%), serine, tyrosine and leucine are also found at position +1. The −2 and −3 positions in the case of the prepilin are usually lysine and glutamine, respectively, the +5 is glutamic acid and the N-terminus of the mature pilin is extremely hydrophobic. The sequence Gly^{-1}-Phe/Met-Thr-Leu-Ile/Leu-Glu^{-5} is conserved among all type IV prepilins. Except for the −1 glycine, single and multiple amino acid substitutions in the leader peptide and amino-

terminal conserved region of the type IV prepilin from *P. aeruginosa* generally had a surprisingly lack of effect on subsequent processing by the prepilin peptidase (MacDonald et al. 1993; Strom and Lory 1992; Strom and Lory 1991). Substitutions of the –1 glycine with almost any other amino acid inhibited cleavage of the leader peptide and prevented assembly of the pilus. Only alanine in place of glycine allowed even partial processing of prepilin (Strom and Lory 1991). Substitutions of the conserved –2 lysine as well as several other residues in the short leader peptide did not affect subsequent proper processing by the prepilin peptidase. Furthermore, substitutions of the +1 phenylalanine with either a polar, hydrophobic or charged residue did not affect the posttranslational processing of the prepilin and neither did the majority of other amino acid substitutions in the highly conserved N-terminal region of the pilin. This flexibility may suggest that the cleavage reaction may occur, although at lower catalytic rates, even when substrates are bound at a relatively low affinity to the active site. The basic leader peptide likely anchors the prepilin in the cytoplasmic membrane, exposing the N-terminus for cleavage and subsequent *N*-methylation (Ado-Met, the methyl donor, is a cytoplasmic molecule). It is possible that removal of the leader peptide and *N*-methylation are necessary prerequisites for the recognition of these proteins by assembly factors during the maturation of the pilus organelle.

The prepilin peptidase is also responsible for the processing of several other substrates (termed pseudopilins) which are necessary for secretion of proteins in Gram-negative bacteria via the general secretion pathway (Nunn and Lory 1992). The pseudopilins, in general, share a number of the conserved features of type IV prepilins including short positively charged leader peptides, the –1 glycine, the +5 glutamic acid

and the highly hydrophobic N-terminal domain. The –2 and –3 positions are usually lysine and glutamine like the prepilins while the +1 phenylalanine position is often different.

Research objectives

The objectives of this study were three-fold. First, preliminary data on the characterization of the non-flagellated *M. voltae* P-2 mutant suggested that a putative preflagellin peptidase was located on the polycistronic transcript encoding the FlaB flagellins. Sequencing of the flagella gene region in *M. voltae* had been previously completed to the end of *flaI*. ORFs homologous to *M. voltae flaC* to *flaI* as well as additional ORFs past *flaI* have been identified upon investigation of the recently published, completed genome sequence of *M. jannaschii*. Consequently, the first priority of this study was to complete the sequencing of putative flagella accessory genes downstream of *flaI* and to the end of the transcript.

Second, experiments to demonstrate preflagellin peptidase activities in *M. voltae* and other methanogens, including other mesophiles (*M. deltae*, *M. maripaludis*, *M. vannielii*, *Methanoculleus marisnigri* and *Methanogenium cariaci*), a thermophile (*M. thermolithotrophicus*) and hyperthermophiles (*M. jannaschii* and *M. igneus*), were to be developed based on an *in vitro* prepilin peptidase assay system of *P. aeruginosa* (Strom et al. 1994). This would be the first report of a preflagellin peptidase.

Third, the preflagellin peptidase assay system was to be modified to determine a set of conditions yielding near maximal *M. voltae* FlaB2 preflagellin cleavage activity. Such an optimized assay would allow us to apply this technology to other research

initiatives in the study of archaeal flagellation. Future work may involve the generation, by PCR, of a family of mutant preflagellins with amino acid substitutions at the conserved positions near the cleavage site, thereby allowing us to determine key residues present in the preflagellin that are required for proper processing.

Chapter 2. Materials and Methods

1. Organisms and Growth Conditions

Methanococcus voltae PS, *Methanococcus jannaschii* JAL-1, *Methanoculleus marisnigri* JR1 and *Methanogenium cariaci* JR1 were obtained from G.D. Sprott (National Research Council of Canada, Ottawa, ON, Canada). *Methanococcus deltae* ΔRC, *Methanococcus maripaludis* JJ and *Methanococcus vannielii* SB were obtained from W.B. Whitman (University of Georgia, Athens, Georgia, USA). *M. voltae*, *M. maripaludis* and *M. deltae* were inoculated at 5% (vol/vol) into 10 ml of Balch medium III (Balch et al. 1979) in 100 ml serum bottles and grown under an atmosphere of CO_2/H_2 (1:4 [vol/vol]) at 37°C with gentle shaking (120 rpm). *M. voltae* P-2, a nonflagellated mutant (Jarrell et al. 1996a), was grown in Balch medium III with 10 μg puromycin (Sigma Chemical Co., St. Louis, MO, USA) per ml. *M. vannielii* was similarly cultured in Balch medium III modified to contain only 4 g/L NaCl. *M. jannaschii* and *M. igneus* (DSM 5666, obtained from the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were grown at 80°C according to Ferrante *et al.* (1990). *M. marisnigri* and *M. cariaci* were grown statically in Balch medium III at room temperature.

Bacterial strains and plasmids are listed in Table 4. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth (Sambrook et al. 1989) at 37°C supplemented with the appropriate antibiotic(s) when necessary. For long-term storage of bacterial cells, 1 ml of an overnight culture was mixed with 70 μl DMSO in 2 ml screw-cap culture tubes and frozen at -70°C.

Table 4. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
DH5α	Host for pUC-based cloning vectors.	K. Poole
BL21	Non-expression host	K. Poole
BL21(DE3)	General purpose expression[a] host	K. Poole
BL21(DE3)pLysS	High-stringency[b] expression host; Cam^r	K. Poole
Plasmids		
pUCBM21	High copy plasmid encoding β-galactosidase; Amp^r	Boehringer Mannheim
pET23a+	Cloning/expression vector; Amp^r	Novagen
pSJS1240	Harbors the *argU* and *ileY* genes encoding tRNAs rare to E. coli	Kim et al (1998)
pKJ91	pT7-7 with the *flaB2* gene inserted at the NdeI site; same orientation as the T7 promoter	Kalmokoff and Jarrell (1991)
pKJ202	pET23a+ with the *flaB1* gene inserted at *Nde*I and *Xho*I sites and fused to a C-terminal polyhistidine tag	This study
pKJ189	pUCBM21 containing *flaJ* as a 2.2 kb *Eco*RI/*Hin*dIII fragment	This study

[a]Expression means that the strain is a λDE3 lysogen, i.e., it carries the gene for T7 polymerase under *lacUV5* control. It is therefore suited to expression from T7 promoters.
[b]High-stringency means that the strain carries pLysS, a pET-compatible plasmid that produces T7 lysozyme, thereby reducing basal expression of target genes.
Cam^r, chloramphenicol resistance; Amp^r, ampicillin resistance.

2. Molecular Biology Techniques

2.1. Chromosomal DNA extraction

Chromosomal DNA was isolated from the *Methanococcus* spp. using the method of Gernhardt et al. (1990) with minor modifications. Ten ml of cells were harvested at 5,000 ×g (Sorvall RC2-B, Ivan Sorvall Inc., Newton, CONN, USA) for 10 min and resuspended in 200 µl spent medium. The subsequent addition of 400 µl TE buffer (100 mM Tris-HCl pH 7.5, 10 mM EDTA) and 6 µl 10% (wt/vol) SDS stock was used to lyse the cells. The viscous lysate was then treated with 6µl RNase (10 mg/ml stock, previously boiled for 10 min to destroy DNase activity) at 37°C for 30 min, followed by the addition of 8 µl proteinase K (10 mg/ml stock) and incubation at 50°C for 45 min. The lysate was extracted twice with an equivalent volume of phenol chloroform (1:1) and once with an equal volume of chloroform. DNA was precipitated by the addition of 1/10 volume of 5 M sodium acetate and 2 volumes of cold 95% ethanol on ice for 1 h. DNA was pelleted at 16,000 ×g (Eppendorf Centrifuge 5415, Brinkmann Instruments Inc., Westburg, NY, USA) for 10 min at 4°C, rinsed with 100 µl 70% ethanol and resuspended in 100 µl sterile distilled water.

2.2. Plasmid extraction

Plasmid was isolated by the Promega Wizard Miniprep system according to the manufacturer's instructions (Fisher Scientific, Nepean, ON, Canada) or by the standard alkaline lysis procedure described by Sambrook et al. (1989). In the standard alkaline lysis method, 1.5 ml of bacterial cells were collected by centrifugation at 16,000 ×g (Eppendorf Centrifuge 5415) for 2 min and resuspended in 100 µl GTE solution (25 mM

Tris-HCl pH 8.0, 50 mM glucose, 25 mM EDTA) and lysed by the addition of 150 µl fresh 1% (wt/vol) SDS/0.2 N NaOH, followed by the addition of 200 µl 3 M potassium/5 M acetate solution. Cellular debris was pelleted at 16,000 ×g for 5 min and the supernatant was transferred to a fresh tube. Plasmid DNA was precipitated by the addition of 0.7 volume room temperature isopropanol and pelleted at 16,000 ×g for 10 min. The pellet was carefully rinsed with 0.5 ml 70% ethanol, air dried and resuspended in 20 µl sterile distilled water.

When plasmid was extracted for use in sequencing reactions, a QIAfilter Plasmid Midi Kit (Qiagen Inc., Mississauga, ON, Canada) was used as per manufacturer's instructions for the isolation of high-copy plasmids (e.g., pUCBM21).

2.3. DNA manipulations

When necessary, two methods of DNA quantitation were used: 1) the absorbance reading at a wavelength of 260 nm for plasmid or chromosomal DNA extractions (at this wavelength, 50 µg/ml of DNA gives an absorbance of 1); or 2) estimation of DNA concentration by comparing the intensity of the sample with the intensity of molecular weight standards of known concentration.

Restriction enzymes and ligase were purchased from Life Technologies (Gibco-BRL, Burlington, ON, Canada) or New England BioLabs Ltd. (Mississauga, ON, Canada). These enzymes were used according to their manufacturer's instructions. When necessary, enzyme was heat inactivated at 65°C for 20 min. Ligation reactions were incubated in a 14°C water bath overnight.

2.4. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR; [Saiki et al. 1998]) was performed in a MiniCycler (MJ Research, Watertown, Mass., USA) using 2.5 units of *Taq* DNA polymerase (5 U/µl; Life Technologies) or *Pwo* DNA polymerase (5 U/µl; Boehringer Mannheim, Laval, PQ, Canada) in 50 µl of a mixture containing 1× supplied buffer with 2 mM $MgSO_4$ (final concentration), 200 µM dNTPs, 50 pmol of each primer and a variable amount of DNA template (10 to 100 ng plasmid or 100 to 750 ng genomic).

DNA was initially denatured at 95°C for 5 min, followed by 30 cycles of 45 sec at 95°C for denaturation, 45 sec at the annealing temperature (usually a few degrees lower than the T_m of the primers being used [Table 5]) and a 72°C extension time which varied according to the size of the fragment to be amplified (approximately 1 min/kb). A final prolonged extension time of 5 min at 72°C followed the 30 cycles. Amplifications were on occasion performed with varying final $MgCl_2$ concentrations of 2, 2.5, 3 and 3.5 mM and with or without the addition of 5% (vol/vol) final concentration of dimethyl sulfoxide (DMSO).

The His-tag pET vectors are a family of expression vectors that use the T7 phage RNA polymerase and T7-specific promoters to express foreign genes in *E. coli*. *M. voltae flaJ* was cloned into the multiple cloning site of the pET23a+ vector at *Nde*I and *Xho*I sites (sites which are absent in *flaJ*), creating an in frame fusion with a polyhistidine (His-tag) sequence corresponding to the C-terminal end of the protein. To do so, forward (5'GGAATTCCATATGATTTTAGATATACTACC [Cortec #3803]) and reverse (5'CCGCTCGAGAACTGGTATTGTAGC [Cortec #3804]) primers were designed to amplify *flaJ*, as well as add a 5' *Nde*I site (single underline) and a 3' *Xho*I site (double underline).

Table 5. Primer pairs used in this study (5' to 3')

Cortec #	Forward Primer	Tm (°C)	Cortec #	Reverse Primer	Tm (°C)	ORF (approximate size)
1778	GAATGGCTTATTCTATTGATAGC	56.2	1779	TTGCTTCTGGGAATCTAGTTCG	55.0	*M. voltae flaJ*[a]
3803	GGAATTCCATATGATTTTAGATATACTACC[b]	62.3	3804	CCGCTCGAGAACTGGTATTGTAGC	57.3	*M. voltae flaJ* (1.7 kb)
4474	GATTATGTTTGGGTATCGATGG	55.0	4475	GGAACAACAAATGGTATAGCAGG	56.2	*M. jannaschii flaK* (500 bp)
4989	GGAATTCCATATGAACATAAAAGAATT[b]	59.2	4990	CCGCTCGAGTTGTAATTCAACAACTT	59.2	*M. voltae flaBI* (650 bp)
3646	TGGGCGATTCTGTAAGTGC	59.2	5466	GATAATCTTCTATGTAATGC	52.3	*M. voltae flaJ* (100 bp)
1778	GAATGGCTTATTCTATTGATAGC	56.2	5466	GATAATCTTCTATGTAATGC	52.3	*M. voltae flaJ* (630 bp)
5559	GGAATTCCATATGGCTTATTCTATTGATAGC[b]	62.9	3803	CCGCTCGAGAACTGGTATTGTAGC	57.3	*M. voltae flaJ* (1.2 kb)
6149	ATTGGGTCAACTTGCAGAGG	57.3	5559	CAAGTTGTGCTATATAATTC	52.3	*M. voltae flaJ* (640 bp)

[a]Designed to amplify a 5' segment of *M. voltae flaJ* by inverse PCR.
[b]Designed to incorporate a 5' *Nde*I site (single underline) for cloning into pHZ36a1, downstream of a T7 promoter.
[c]Designed to incorporate a 3' *Xho*I site (double underline) for cloning into pHZ36a1, creating an in frame fusion with a polyhistidine (His tag) sequence corresponding to the C terminal end of the protein.

The *Nde*I site created an ATG codon seven bases downstream of a ribosome binding site on the pET vector. Amplification of *M. voltae flaJ* with these primers was performed as follows: 95°C for 5 min; 30 cycles of 95°C for 45 sec/50°C for 45 sec/72°C for 2 min; and 72°C for 5 min.

Amplification of a ~ 500 bp piece of *M. jannaschii* ORF MJ0902 (which would correspond to a putative *flaK* in *M. voltae*), for use as a DNA probe, was performed using the primers 5'GATTATGTTTGGGTATCGATGG (Cortec #4474) and 5'GGAACAACAAATGGTATAGCAGG (Cortec #4475) at an annealing temperature of approximately 50°C and an extension time of 45 s.

M. voltae flaB1 was cloned into pET23a+. PCR amplification of *flaB1* was performed using pKJ43, which contains a 2 kb *Pst*I fragment encompassing *flaB1* (Kalmokoff and Jarrell 1991), as template and the primers 5'GGAATCCATATGAACATAAAAGAATT (Cortec #4989) and 5'CCGCTCGAGTTGTAATTCAACAACTT (Cortec #4990), which were designed to incorporate a 5' *Nde*I site (single underline) and a 3' *Xho*I site (double underline). *flaB1* was amplified using the following program: 95°C for 5 min; 30 cycles of 95°C for 45 sec/50°C for 45 sec/72°C for 2 min; and 72°C for 5 min.

Primer dimers were removed by passage of the reaction through a Qiaquick spin PCR purification column (Qiagen Inc.).

2.5. Inverse PCR

Inverse PCR amplification of nucleotide sequence downstream of *M. voltae flaI* involved the identification of a sticky end restriction endonuclease site near the 3' end of

the known DNA sequence (*flaI*) and synthesis of divergent primers (5'GAATGGCTTATTCTATTGATAGC [Cortec #1778] and 5'TTGCTTCTGGGAATCTAGTTCG [Cortec #1779]) complementary to a region between that site and the extreme 3' end. Genomic DNA at a concentration of approximately 150 µg/ml from *M. voltae* was digested with *Eco*RI at 37°C for 2 h. The restriction endonuclease was heat inactivated and removed by passage of the reaction through a Qiaquick spin PCR purification column (Qiagen Inc.). Digestion fragments were separated by agarose gel electrophoresis and a 6 kb digestion fragment was identified by Southern hybridization. The DNA fragment of interest was then purified from agarose by the "Prep-A-Gene" method as described below (section 2.7). Religation of the 6 kb *Eco*RI fragment was performed at an approximate concentration of 40 µg/ml to optimize circularization (Collins and Wessiman 1984). Amplification was performed (as previously described in section 2.4) at an annealing temperature of 50°C and an extension time of 8 min. Amplification of this large template was also performed using the Expand™ Long Template PCR System (Boehringer Mannheim) according to the manufacturer's instructions.

2.6. DNA electrophoresis

Electrophoresis of DNA was performed at constant voltage (Bio-Rad Model 500/200 Power Supply, Bio-Rad Laboratories, Hercules, CA, USA) in a MINNIE Submarine Agarose Gel Unit Model HE 33 (Hoefer Scientific Instruments, San Francisco, CA, USA) with 0.8% (wt/vol) agarose (ICN Biomedicals Inc., Auroroa, Ohio, USA) gels in Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 0.6% [vol/vol] glacial acetic

acid, 5 mM EDTA pH 8.0 [Sambrook et al. 1989]). DNA samples were loaded in the presence of 1× DNA loading buffer (48% sucrose, 0.25% bromophenol blue, 10 mM EDTA). Markers used were either *Hin*dIII- or *Hin*dIII/*Eco*RI-digested λ DNA (17.4 µl [8 µg] λ DNA, 18 µl 10× restriction enzyme buffer, 2.5 µl *Hin*dIII, 2.5 µl *Eco*RI, 139.6 µl water; incubate at 37°C for 1 h and add 20 µl 1× DNA loading buffer; final concentration was 40 ng/µl) or 100 bp DNA ladder (8 µl 100 bp stock [Boehringer Mannheim], 72 µl sterile distilled water, 20 µl 1× DNA loading buffer). Ethidium bromide was added to unpolymerized gels to achieve a final concentration of 1 µg/ml and DNA was visualized under ultra violet light.

2.7. Purification from agarose

The extraction of plasmid or chromosomal DNA from agarose gels was performed using the "Prep-A-Gene" method (Bio-Rad). The band of interest was excised (200 µl approximate volume) with a sterile razor and placed in a sterile microfuge tube and dissolved in 600 µl (3× volume) of binding buffer (6 M $NaClO_4$, 50 mM Tris, 10 mM EDTA pH 8.0) at 50°C. DNA was then bound to a silica-based matrix (10 to 15 µl) for 5 to 10 min at room temperature. After a brief centrifugation (30 sec), the pellet was washed twice with 25× matrix volume (250 to 375 µl) of wash buffer (0.8 M NaCl, 40 mM Tris, 4 mM EDTA pH 7.4) and eluted twice with 10 µl sterile distilled water.

2.8. Transformation

2.8.1. Heat shock

Competent *E. coli* DH5α were prepared as described by Inoue et al. (1991). A 5 ml starter culture was grown at 37°C and inoculated into 250 ml of LB medium (in a 1 L flask) and incubated with shaking at 18°C until $O.D._{600}$ ~ 0.6 (~ 36 h). The cells were then placed on ice for 10 min and harvested by centrifugation at 2,500 ×g for 10 min at 4°C. The pellet was resuspended in 80 ml of ice cold transformation buffer (10 mM Pipes [Piperazine-N,N'-bis{2-ethan-sulfonic acid}], 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$) and centrifuged as above. The pellet was then resuspended in 20 ml of transformation buffer and supplemented with 7% (vol/vol) DMSO. The cell suspension was incubated on ice for 10 min and dispensed as 1 ml aliquots in 2 ml screw cap tubes for storage at -80°C.

On ice, 0.5 to 2 µl of a ligation reaction was added to 100 µl of competent *E. coli* DH5α in a chilled polypropylene tube for 30 min. These cells were heated at 37°C for 45 s and placed on ice for 2 min before 950 µl of room temperature LB medium was added. Transformed cells were incubated for 1 h at 37°C with shaking (225 rpm). One hundred µl was plated on LB containing the appropriate antibiotic. The balance of the transformation reaction was pelleted, resuspended in 100 µl of spent medium and plated.

2.8.2. Electroporation

For preparation of electro-competent cells, 1 L of rich broth appropriate for rapid cell growth was inoculated with 1/100 volume of fresh overnight culture. Cells were grown to early to mid log phase ($O.D._{600}$ ~ 0.5) and the growth flask was chilled on ice for 15 to 30 min. Cells were harvested by centrifugation in a cold rotor at 4,000 ×g

(Sorval RC2-B) for 15 min. The pellet was resuspended in a total of 1 L of cold sterile distilled water. The cells were pelleted as above, resuspended in 0.5 L of cold sterile distilled water and pelleted again. To store cells at -70°C, cells were resuspended in 20 ml of cytoprotectant (15% [vol/vol] glycerol) and centrifuged as above. Cells were resuspended in a final volume of 2 to 3 ml of cytoprotectant and 50 µl aliquots were divided into tubes for long-term storage.

Electro-competent cells (50 µl) and DNA preparation were mixed in a 0.2 cm cuvette (Bio-Rad) and electroporations were performed with a Gene Pulser (Bio-Rad) using the following conditions: 200 Ω of resistance, 2.5 kV of current intensity and 25 µFD of capacitance. After electroporation, cells were allowed to recover in 800 µl SOC medium (Sambrook et al. 1989) at 37°C for 1 h. Fifty µl were then plated on LB containing the appropriate antibiotic.

When the DNA preparation to be electroporated was the product of a ligation, a Qiaquick spin PCR purification column (Qiagen Inc.) was used to remove salts from the mixture.

2.9. Southern hybridization

2.9.1. DNA transfer

Electrophoresed plasmid or chromosomal DNA digestion fragments were transferred from agarose gels onto positively charged nylon membrane (Boehringer Mannheim) according to manufacturer's instructions. The agarose gel was immersed in denaturation solution (0.5 N NaOH, 1.5 M NaCl) for 45 min, neutralization solution (0.5 M Tris-HCl pH 7.5, 3 M NaCl) for a subsequent 45 min and 10× standard saline citrate

(SSC) buffer diluted from 20× SSC stock solution (3 M NaCl, 300 mM sodium citrate pH 7.0) for 10 min. DNA was blotted to the membrane overnight by capillary transfer using 10× SSC buffer. DNA was then fixed on the membrane by baking at 80°C for 2 h.

2.9.2. Colony hybridization

Colony hybridizations were used to allow rapid screening of bacterial populations for specific DNA sequences. Colonies on agarose plates were cooled for 30 min at 4°C. Nylon membrane discs (Boehringer Mannheim) were gently placed onto the surface of the agarose plate and left in place for 1 min. The membrane discs were carefully removed and briefly blotted on dry blotting paper. The membrane discs were then placed (colony side up) for 15 min on 2 layers of filter paper presoaked with denaturation solution, briefly blotted, placed for 15 min on filter paper presoaked with neutralization solution, briefly blotted and placed for 10 min on filter paper presoaked with 2× SSC. The membranes were then baked dry for at least 30 min at 80°C and treated with Proteinase K to digest interfering proteins. 0.5 ml of 2 mg/ml Proteinase K was pipeted on each disc and incubated for 1 h at 37°C. Cellular and agar debris were removed by blotting the membranes between filter paper fully wetted with sterile distilled water.

2.9.3. Preparation of probes

Oligonucleotides were 3'-end labelled with the nonradioactive steroid hapten digoxigenin (DIG) using the Genius™ 5 Oligonucleotide 3'-End Labeling Kit (Boehringer Mannheim) as per manufacturer's instructions. Approximately 100 pmol of oligonucleotide was added to 4 µl 5× reaction buffer and 4 µl $CoCl_2$ solution. To this mixture, 1 µl of DIG-ddUTP and 1 µl of terminal transferase was added and the reaction volume was increased to 20 µl with sterile distilled water. The reaction was incubated at

37°C for 15 min, chilled on ice and terminated by the addition of 1 µl of 200 mM EDTA (pH 8.0).

The random primed method was used to DIG label templates of 0.1 to 10 kb. A DIG DNA Labeling and Detection Kit (Boehringer Mannheim) was used. One to three µg of DNA was diluted in 15 µl of sterile distilled water, heat denatured in a boiling water bath for 10 min and quickly chilled on ice. Two µl of 10× hexanucleotide mixture and 2 µl of 10× dNTP labelling mixture were added to the tube (on ice). Finally, 1 µl of Klenow enzyme (2 U) was added and the reaction was incubated at 37°C for at least 1 h. Longer incubations (overnight) increased the yield of DIG-labeled DNA. The reaction was stopped by the addition of 2 µl of 200 mM EDTA (pH 8.0).

2.9.4. Prehybridization, hybridization and stringency washes

The membrane was prehybridized in a sealed hybridization bag containing 20 ml prehybridization solution (5× SSC, 0.1% [wt/vol] N-lauroylsarcosine, 0.02% [wt/vol] SDS, 1% [wt/vol] blocking reagent (from 10% blocking reagent stock solution, Boehringer Mannheim]) per 100 cm^2 for at least 1.5 h at the hybridization temperature (usually a few degrees lower than the T_m of the specific probe).

The membrane was subsequently hybridized overnight in 20 ml hybridization solution (prehybridization solution containing 5 to 25 ng/ml DNA probe) per 100 cm^2 at the same temperature. Double-stranded DNA probes were diluted in 100 µl hybridization solution and denatured by heating in a boiling water bath for 10 min. Oligonucleotide probes did not require denaturation.

Post-hybridization stringency washes were performed 5 min twice at room temperature with ample 2× SSC containing 0.1% (wt/vol) SDS and 15 min twice at 50°C with 0.1× SSC containing 0.1% (wt/vol) SDS.

2.9.5. Probe detection

Chemiluminescent detection was performed using the DIG Luminescent Detection Kit with CSPD® (25 mM disodium 3-(4-methoxyspiro{(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.^{3,7}]decan}-4-yl) phenyl phosphate) (Boehringer Mannheim). The membrane was briefly rinsed with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl; pH 7.5) and followed by two 30 min incubations at room temperature. The first was with 20 ml of 1× blocking buffer (10× blocking buffer stock diluted with maleic acid buffer) and the second with 20 ml 1× blocking buffer containing 2 µl (1:10,000) anti-DIG antibody (Boerhringer Mannheim). The membrane was then washed 15 min twice at room temperature in a copious volume of washing buffer (maleic acid buffer with 0.3% [vol/vol] Tween 20), equilibrated for 2 to 5 min at room temperature in 20 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5) and incubated in darkness for 5 min in 1 ml of detection buffer containing 10 µl CSPD®. The excess liquid was subsequently removed and the membrane was incubated at 37°C for a further 5 to 15 min to enhance the luminescent reaction before exposure to autoradiography film (Scientific Imaging Film X-Omat Blue XB-1, Mandel Scientific Company Ltd., Guelph, ON, Canada)

2.9.6. Membrane stripping

To remove probe, membranes were thoroughly rinsed in water, incubated twice in 0.2 M NaOH, 0.1% (wt/vol) SDS at 37°C for 20 min, washed 10 min in 2× SSC and dried or kept wet for immediate prehybridization.

2.10. DNA sequencing and oligonucleotide synthesis

All DNA sequencing and oligonucleotide synthesis was performed by Cortec DNA Services Laboratories Inc. (Queen's University, Kingston, ON, Canada).

2.11. RNA isolation

Total RNA was isolated from *E. coli* using the Qiagen RNeasy Mini Kit (Qiagen Inc.) according to manufacturer's 'RNA isolation from bacteria' protocol.

Total RNA was isolated from *Methanococcus* spp. using the Qiagen RNeasy Mini Kit with minor modifications to the 'RNA isolation from bacteria' protocol. Ten ml of overnight methanogen culture was anaerobically transferred to a 10 ml tube modified to accept a serum bottle stopper. Cells were harvested anaerobically under CO_2/H_2 by centrifugation at 5,000 ×g (Sorvall RC2-B) for 10 min at 4°C and resuspended in 450 µl TE/RLT buffer (100 µl TE buffer and 350 µl buffer RLT). The addition of lysozyme is unnecessary when isolating RNA from archaea. The RNA isolation protocol was continued as described in the RNeasy Mini Handbook (Qiagen Inc.). When necessary, residual DNA was removed from the final RNA preparation by treatment with RQ1 RNase-Free DNase (Promega Corp., Madison, WI, USA) as per manufacturer's instructions.

2.12. Formaldehyde agarose (FA) gel electrophoresis

Electrophoresis of RNA was performed at 100 V in a Horizontal Gel Electrophoresis Unit (Bethesda Reasearch Laboratories, Life Technologies Inc.) for 2 to 3 h with 1.2% Formaldehyde-Agarose (FA) gels in 1× FA gel running buffer (100 ml 10× FA gel buffer [200 mM 3-{N-Morpholino}propanesulfonic acid {MOPS}, 50 mM sodium acetate, 10 mM EDTA {pH 7.0}], 20 ml 37% [vol/vol] formaldehyde, 880 ml RNase-free water [1 L of distilled water was treated with 1 ml of DEPC, stored at room temperature overnight and then autoclaved before use]). FA gels were equilibrated in 1× FA gel running buffer for at least 30 min and pre-run for at least 10 min before the addition of RNA samples. Samples were prepared by the addition of 1 volume of 5× RNA loading buffer (16 µl saturated bromophenol blue, 80 µl 500 mM EDTA [pH 8.0], 720 µl 37% [vol/vol] formaldehyde, 2 ml 100% glycerol, 3084 µl formamide, 4 ml 10× FA gel buffer; add supplied RNase-free water to 10 ml) per 4 volumes of RNA sample and heated for 10 min at 65°C. Samples were kept on ice before loading. A 0.24 to 9.5 kb RNA ladder (Gibco-BRL, Life Technologies) was used as the molecular size standard.

2.13. Northern hybridization

Electrophoresed RNA samples were transferred in 20× SSC buffer from FA gels to positively charged nylon membranes. The remaining steps (prehybridization, hybridization, stringency washes and probe detection) are the same as those described for Southern hybridization (sections 2.9.4. and 2.9.5.).

Oligonucleotide and DNA probes were labelled with DIG using the Genius™ Nonradioactive Nucleic Acid Labelling and Detection System (Boehringer Mannheim) as described above for the Southern hybridization protocol.

Primer pairs were designed to amplify fragments of *M. voltae flaJ* for use as DNA probes. 5'TGGGCGATTCTGTAAGTGC (Cortec #3646) and 5'GATAATCTTCTATGTAATGC (Cortec #5466) and 5'ATTGGGTCAACTTGCAGAGG (Cortec #6149) and 5'CACGTTGTGCCATATAACCG (Cortec #6150) were used to amplify ~ 100 bp and ~ 640 bp fragments of *M. voltae flaJ*, respectively.

2.14. Reverse transcriptase (RT) PCR

RT-PCR technology permits the amplification of DNA from single-stranded RNA, DNA or an RNA:DNA hybrid using a mixture of reverse transcriptase and DNA polymerase enzymes. In this study, this system was be used to detect the presence of mRNA containing *flaJ*.

RT-PCR was performed in a MiniCycler (MJ Research, Watertown, Mass., USA) using 5 U of SuperScript II RT/*Taq* Mix (5 U/μl; Life Technologies) in 50 μl of a mixture containing 2× supplied reaction mix, 50 pmol of each primer and a variable amount (100 to 750 ng) of RNA template.

Amplification of a ~ 630 bp segment of *flaJ* was performed using the primers 5'GAATGGCTTATTCTATTGATAGC (Cortec #1778) and 5'GATAATCTTCTATGTAATGC (Cortec #5466). First strand cDNA synthesis was amplified at 50°C for 25 min. The RNA:DNA hybrid was then denatured at 94°C for 2 min. The cDNA was then amplified by 35 cycles of 15 sec at 94°C for denaturation, 30 sec at 50°C for annealing and an

extension time of 1 min at 72°C. A final prolonged extension time of 10 min at 72°C followed the 35 cycles.

Amplification of *flaB1* from total RNA isolated from *M. voltae* was performed using the primers 5'GGAATCCATATGAACATAAAAGAATT (Cortec #4989) and 5'CCGCTCGAGTTGTAATTCAACAACTT (Cortec #4990), also at an annealing temperature of 50°C and extension time of 1 min.

3. Protein Techniques

3.1. Isolation

3.1.1. Whole cells

To prepare whole cell lysates, 1 ml of archaeal or bacterial culture was pelleted, resuspended in 50 µl distilled water and 50 µl 2× electrophoresis sample buffer (ESB; 0.0625 M Tris pH 6.8, 1% [wt/vol] SDS, 10% [vol/vol] glycerol, 2% [vol/vol] 2-mercaptoethanol, 0.001% [wt/vol] bromophenol blue) and boiled for 5 min.

3.1.2. Bacterial envelopes

Cells were harvested by centrifugation at 6,000 ×g for 15 min, washed once in 100 mM Tris-HCl (pH 6.8) containing 10% (wt/vol) sucrose, then spheroplasted (Thome and Müller 1991) by addition of 100 mM Tris-HCl (pH 6.8) containing 18% (wt/vol) sucrose, 100 µg/ml lysozyme and 4 mM ethylenediamine tetraacetic acid (EDTA). Spheroplasts were then harvested at 10,000 ×g for 15 min and lysed by resuspension in water and brief sonication (45 sec). Unbroken cells were removed by low speed centrifugation (3,500 ×g for 5 min). Crude membrane fragments were then harvested at

16,000 ×g for 30 min, resuspended in sterile distilled water and stored at -20°C. Samples to be analyzed by SDS-PAGE were diluted 1:1 in 2× ESB and boiled for 5 min.

3.1.3. Methanogen envelopes

Ten ml of methanogen cells were grown overnight to late-exponential phase as previously described, then harvested by centrifugation at 16,000 ×g (Eppendorf Centrifuge 5415) for 5 min and resuspended in 100 µl of the overnight culture. Crude cell envelopes were prepared by lysing the cells in 1.4 ml sterile distilled water, the resulting envelopes were then isolated by centrifugation at 16,000 ×g for 10 min and resuspended in 100 µl of sterile distilled water. All isolations were performed aerobically. Samples to be analyzed by SDS-PAGE were diluted 1:1 in 2× ESB and boiled for 5 min.

3.2. Protein quantitation

Spectophotometric determination of protein concentration was performed using the Pierce BCA™ Protein Assay (Pierce, Rockford, IL, USA).

3.3. Protein expression

3.3.1. Induction

Plasmid was isolated from *E. coli* DH5α cells containing the pET23a+/foreign gene construct and transformed into *E. coli* BL21(DE3), *E. coli* BL21(DE3) containing pLysS, or *E. coli* BL21(DE3) containing pSJS1240 (pSJS1240 harbors the argU and ileX genes encoding rare tRNAs [$tRNA_{AGA/AGG}$ and $tRNA_{AUA}$] [Kim et al. 1988]). A single colony from the streaked plate of transformants was inoculated into a starter culture of 2

ml and incubated at 37°C for 4 to 5 h (in a 10 mm glass culture tube) with vigorous shaking (300 rpm) and stored overnight at 4°C. The next day, the cells were pelleted and resuspended in 2 ml fresh medium with appropriate antibiotics and inoculated into 50 ml fresh medium and grown to an $O.D._{600}$ of 0.5 to 0.6. Isopropylthio-β-D-galactosidase (IPTG; Life Technologies) was added to 0.4 mM and growth was allowed to continue for approximately 3 h. For analysis, 0.5 ml samples were removed before induction and at 1 h intervals, examined by spectophotometric analysis ($O.D._{600}$) and total cell lysates of uninduced and induced cultures were analyzed by SDS-PAGE and stained with Coomassie Blue G-250 (section 3.5.) to identify the presence of induction products.

Before induction, 4 control plates were prepared to test the fraction of cells able to express the target gene: 1) 1 mM IPTG (assuming the plate volume was 25 ml, 4 µl of 200 mg/ml IPTG stock was diluted in 100 µl LB medium, plated with beads and allowed to absorb for 5 to 10 min); 2) 1 mM IPTG and antibiotic(s); 3) antibiotic(s); and 4) no IPTG or antibiotic(s). A 10^{-5} dilution was prepared from the 2 ml starter culture and 100 µl was plated on each control plate.

3.3.2. Affinity chromatography

Ni^{2+} affinity chromatography was used to purify proteins expressed by the pET system as per manufacturer's instructions (Novagen, Madison, WI, USA). To prepare the cell extract, 50 to 100 ml of cells were harvested by centrifugation at 5,000 ×g (Sorval RC2-B) for 5 min. For purification under non-denaturing conditions, the cells were resuspended in 4 ml ice-cold binding buffer. The solution was briefly sonicated (in a tube on ice) to shear the DNA and centrifuged at 39,000 ×g for 20 min to remove cellular debris.

For purification under denaturing conditions, 6 M urea was added directly to the concentrated binding buffer, wash buffer and elution buffer. Cells were resuspended in 40 ml 1× binding buffer, briefly sonicated and centrifuged at 20,000 ×g for 15 min to collect inclusion bodies and cellular debris. The pellet was then resuspended in 20 ml 1× binding buffer by sonication and centrifugation at 20,000 ×g was repeated. Five ml 1× binding buffer (with denaturant) was used to resuspend the pellet and the solution was incubated on ice for 1 h to completely dissolve the protein. Any remaining insoluble material was removed by a final centrifugation at 39,000 ×g for 20 min.

3.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Samples were loaded onto 0.75 mm thick gel slabs in a miniature gel electrophoresis apparatus (Mighty Small II SE 250, Hoefer Scientific Instruments, San Francisco, CA, USA) and electrophoresed at 100 mV through a 4.5% stacking gel (0.75 ml 30:0.8 [wt/vol] acrylamide:bis-acrylamide, 2.9 ml sterile distilled water, 1.25 ml 0.5 M Tris [pH 6.7], 50 µl 10% ammonium persulfate [APS], 50 µl 10% SDS and 6 µl TEMED) and 12% resolving gel (3.8 ml 30:0.8 [wt/vol] acrylamide:bis-acrylamide, 2.7 ml sterile distilled water, 2.5 ml 1.5 M Tris [pH 8.8], 60 µl APS, 100 µl SDS and 6 µl TEMED) in Laemmli running buffer (0.025 M Tris, 0.192 M glycine, 0.1% [wt/vol] SDS; pH 8.3). Pre-stained SDS-PAGE low range molecular weight standards (Bio-Rad) used were lysozyme (M_r = 19,400 Da), soybean trypsin inhibitor (M_r = 28,300 Da), carbonic anhydrase (M_r = 33,400 Da), ovalbumin (M_r = 48,300 Da), bovine serum albumin (M_r = 82,000 Da) and phosphorylase B (M_r = 104,000 Da). Protein samples

were mixed (1:1) in 2× protein electrophoresis sample buffer (ESB; 0.0625 M Tris pH 6.8, 1% [wt/vol] SDS, 10% [vol/vol] glycerol, 2% [vol/vol] 2-mercaptoethanol, 0.001% [wt/vol] bromophenol blue).

3.5. Staining of polyacrylamide gels

SDS-polyacrylamide gels were immersed for 1 to 2 min in protein stain (0.04% [wt/vol] Coomassie brilliant blue G-250 [Bio-Rad], 3.5% [vol/vol] perchloric acid) brought to a boil in a microwave and destained in distilled water, also brought to a boil in a microwave (Faguy et al. 1996).

3.6. Western blotting

Electrophoresed proteins were electrophoretically transferred to Immobilon-P nitrocellulose membrane (Millipore, Bedford, Mass., USA) in a TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments) at 225 mA for 45 min immersed in Towbin blotting buffer (5.8 g Tris, 29.0 g glycine, 400 ml methanol, 1.6 L distilled water [Towbin et al. 1979]).

Western blots were performed according to manufacturer's Chemiluminescence Blotting Substrate (POD) protocol (Boehringer Mannheim). Membranes were briefly rinsed in Tris buffered saline (TBS; 7.76 g NaCl, 0.6 g Tris, 1 L distilled water) supplemented with Tween 20 (TBST) (500 µl/L) and subsequently incubated for a minimum of 1 h at room temperature in 50 ml 1% (vol/vol) blocking reagent with gentle rocking. After two 5 min washes with TBST, blots were incubated for 1 h in primary antibody (chicken anti-flagellin at 1:10,000; produced by RCH Antibodies, Sydenham,

ON [Bayley and Jarrell 1999]) diluted in 10 ml of 0.1% (vol/vol) blocking reagent, washed once 15 min and 4× 5 min in TBST and incubated for 1 hr in secondary antibody (peroxidase linked rabbit anti-chicken antibody at 1:50,000; Jackson Immunoresearch Laboratories, West Grove, Pa., USA) diluted in 10 ml 0.1% (vol/vol) blocking reagent. QIAexpress Anti-His (Qiagen Inc.) antibodies were also used for the detection of recombinant proteins containing a 6× His-tag as per manufacturer's instructions. A chemiluminescent detection system designed for use with peroxidase-labeled reporter molecules was used to visualize the proteins. Blots were immediately sealed in hybridization bags and immediately exposed to Kodak X-Omat Blue XB-1 autoradiography film (Mandel Scientific).

If required, the membrane could be stripped and reprobed. To strip, the membrane was incubated in TBS containing 100 mM 2-mercaptoethanol and 2% SDS with gentle shaking for 30 min at 50°C, followed by 2 washes at room temperature for 15 min in a copious volume of TBST. The membrane was then ready for the non-specific blocking step described above.

If staining was required following protein transfer, either Coomassie Blue R250 or Ponceau stain was used. To stain with Coomassie, the membrane was briefly stained with 0.1% (wt/vol) Coomassie Blue R250 and destained in 50% (vol/vol) methanol. To Ponceau stain, the membrane was stained (0.5 g Ponceau S, 1 ml glacial acetic acid, 98.5 ml water) for 3 to 5 min or until bands appear and destained in water or TBST. To completely destain the membrane, the membrane was incubated for at least 10 min under constant agitation in water or TBST.

3.7. N-terminal sequencing

Proteins for N-terminal sequencing were electrophoresed by SDS-PAGE and transferred to Immobilon P as described above. The membrane was briefly stained with 0.1% (wt/vol) Coomassie Blue R250, destained in 50% (vol/vol) methanol and rinsed thoroughly with distilled water. Bands of interest were removed with a sterile blade. Sequencing was performed by David Watson (National Research Council of Canada, Ottawa, ON, Canada).

4. Enzymatic Assay

4.1. Preflagellin peptidase assay

The standard preflagellin peptidase reaction mixture contained ~ 72 µg of induced *E. coli* KJ91 membranes (as substrate) (Figure 3) combined with ~ 18 µg of methanogen membranes (as enzyme source) in a final volume of 60 µl of 25 mM HEPES buffer pH 7.5 containing 0.5% (vol/vol) Triton X-100 (Figure 4). All assays were performed aerobically. Each of the preflagellin peptidase assays were conducted near the optimum growth temperature of the methanogen tested: 37°C for reactions involving a mesophilic archaeon, 60°C for the thermophilic *M. thermolithotrophicus* and 80°C for the hyperthermophiles *M. jannaschii* and *M. igneus*. The reaction was started upon addition of the methanogen membranes and stopped by the addition of 15 µl ESB to 10 µl aliquots (removed at time points of 0, 2, 10 and 30 minutes) and boiling for 5 minutes. A portion of the reaction was analyzed by SDS-PAGE and immunoblotting with anti-flagellin primary antibody.

Figure 3. Schematic representation of the expression of *M. voltae* FlaB2 preflagellin in *E. coli*. *M. voltae* FlaB2 was expressed in *E. coli* using the T7 polymerase system (Tabor and Richardson 1985). $CaCl_2$-competent *E. coli* DH5α were transformed with pT7-7 carrying the PCR-reconstructed *flaB2* gene under the control of a T7 promoter. Plasmid from a single transformant was isolated and transformed into *E. coli* BL21(DE3) containing pLysS. A single colony containing the correct plasmid was purified and designated *E. coli* KJ91. *E. coli* KJ91 was grown overnight then inoculated at 1% (vol/vol) into 50 ml fresh medium with antibiotics and grown to an optical density at 600 nm ($O.D._{600}$) of 0.5 to 0.6. IPTG was added to 2 mM and growth was allowed to continue for an additional 2 to 3 h. Cells were harvested by centrifugation and spheroplasted. Crude membrane fragments were isolated and resuspended in sterile distilled water and stored at -20°C until used as substrate in the peptidase assay.

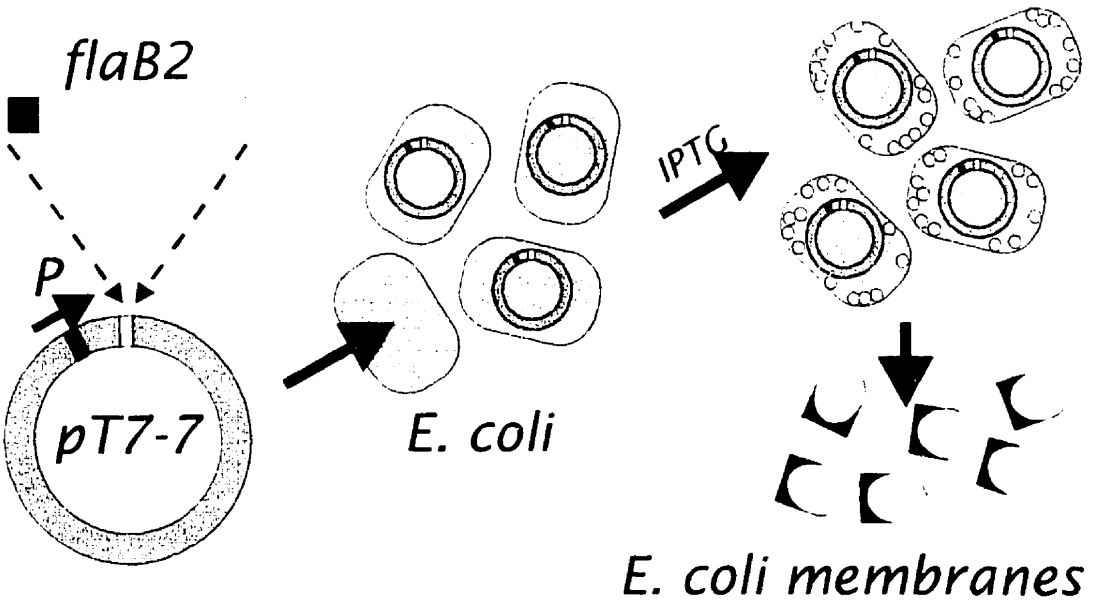
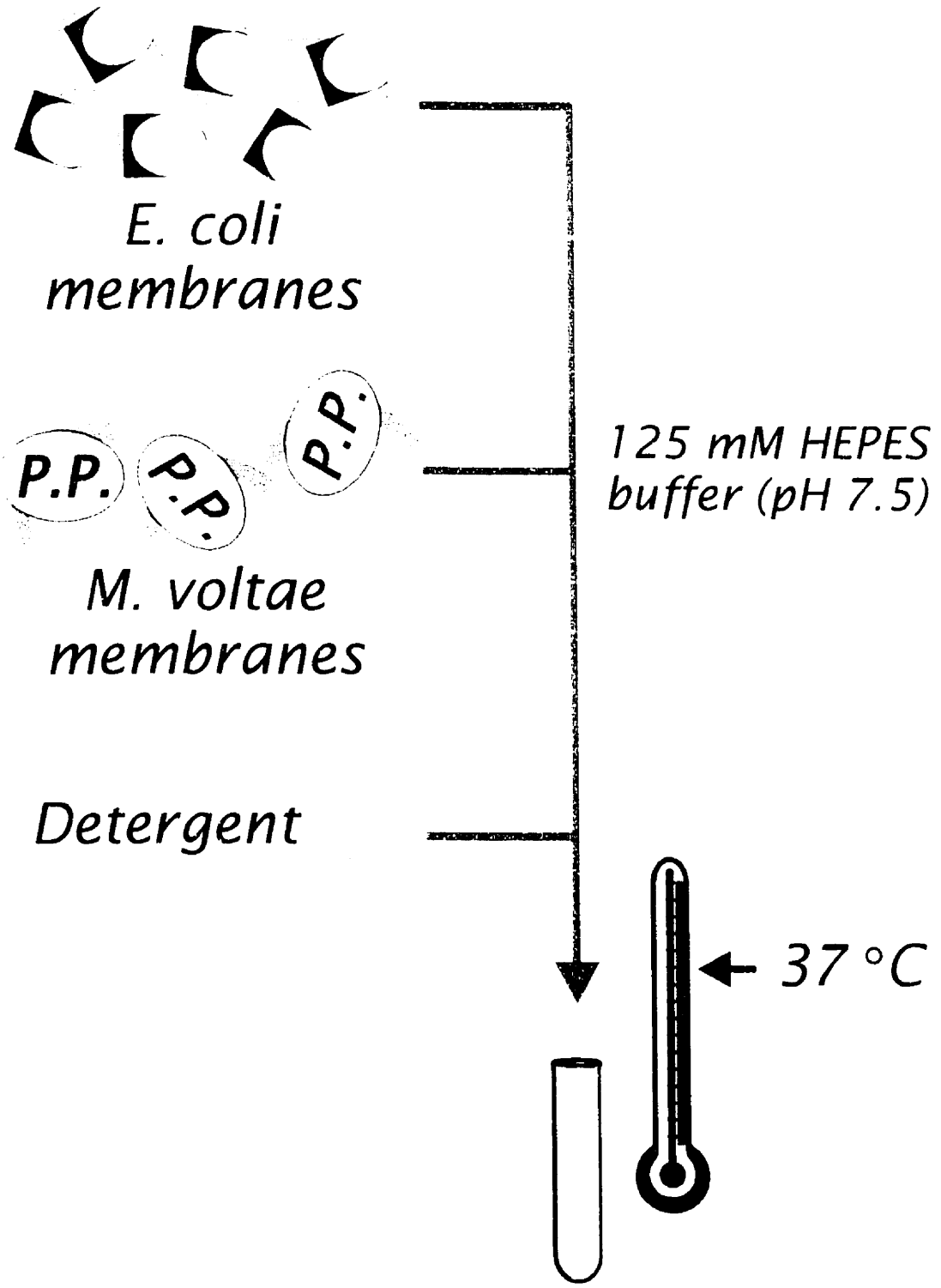

flaB2
P
pT7-7
E. coli
IPTG
E. coli membranes

Figure 4. Schematic representation of the standard preflagellin peptidase assay. The standard preflagellin peptidase reaction was performed with approximately 72 μg of induced *E. coli* membranes (substrate source) combined with approximately 18 μg of methanogen membranes (enzyme source) in 25 mM HEPES buffer (pH 7.5) containing 0.5% (vol/vol) Triton X-100 and at a reaction temperature near the optimal growth temperature of the organism being tested.


E. coli membranes
P.P.
P.P.
P.P.
M. voltae membranes
125 mM HEPES buffer (pH 7.5)
Detergent
37 °C

4.1.1. Determination of conditions yielding maximum *M. voltae* FlaB2 preflagellin peptidase activity

Using *M. voltae* membranes as the enzyme source, a number of independent modifications to the standard preflagellin peptidase reaction conditions were made to evaluate detergent, salt, pH and temperature optima. Although, true optimization was not performed (i.e., by factorial design), these conditions were examined to determine conditions yielding maximum preflagellin peptidase activity. First, the peptidase assay was performed with no detergent and with different final concentrations (0.125-1% [vol/vol]) of Triton X-100. As well, a number of other nonionic detergents were tested at 0.5% (vol/vol) in 25 mM HEPES buffer pH 7.5, including Nonidet P-40 (NP-40), Tween 20, Tween 80 and Brij 58, as well as sodium dodecyl sulfate (SDS) (an alkyl ionic detergent). Second, the necessity of acidic phospholipids for archaeal preflagellin cleavage was tested by running the assay in the absence or presence of 0.5% (vol/vol) cardiolipin (Sigma Chemical Co., St. Louis, MO). Third, the salt concentration of the standard reaction mixture was augmented (0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.2 M) by the addition of KCl or NaCl. Fourth, the influence of pH on the cleavage reaction was examined using MES (pH 5.5 and 6.5), HEPES (pH 7.5 and 8.5) and Bis-Tris Propane (pH 8.5, 9.5 and 10.5) buffers at 25 mM final concentration. Finally, the standard reaction for *M. voltae* preflagellin peptidase activity was conducted at 21, 30, 40, 50 and 60°C.

Chapter 3. Results

1. Genetic and biochemical characterization of putative flagella accessory gene(s) in *M. voltae*

Four flagellin genes (*flaA*, *flaB1*, *flaB2* and *flaB3*) encoded by two transcriptional units have been identified in the mesophilic archaeon *M. voltae* (Kalmokoff and Jarrell 1991). One transcriptional unit (~ 800 bp) contains only *flaA*, the other, polycistronic transcriptional unit (of at least 5.4 kb), contains *flaB1*, *flaB2* and *flaB3* and additional downstream genes which have been presumed to be flagella related (Kalmokoff and Jarrell 1991). Seven ORF's were identified downstream of *flaB3* (designated *flaC* to *flaI*) and were sequenced along with the four flagellin genes. When compared to the recently completed *M. jannaschii* genome project (Bult et al. 1996), *M. jannaschii* ORFs MJ0894 to MJ0900 are similar to *M. voltae flaC* to *flaI*. Furthermore, an additional six ORFs past the *M. voltae flaI* equivalent in *M. jannaschii* (MJ0901 to MJ0906) could be cotranscribed with MJ0894 to MJ0900 before an ORF on the opposite strand is encountered. ORFs past *M. voltae flaI*, if any, have yet to be identified. Characterization of a non-flagellated *M. voltae* mutant (*M. voltae* P-2) had suggested that a putative preflagellin peptidase might be co-transcribed with the *flaB* genes (Jarrell et al. 1996a). The first stage of my research involved the sequencing of additional ORF(s) downstream of *flaI* in *M. voltae* and biochemical characterization of their gene product(s).

1.1. Strategy for cloning putative flagella accessory gene(s) in *M. voltae*

The initial cloning strategy involved the amplification of sequence immediately downstream of *flaI* using inverse PCR technology. The choice of restriction enzyme used

was limited to *Eco*RI, for which a recognition sequence was known to be present near the 3' end of *flaI*. *M. voltae* DNA was digested with *Eco*RI, restriction fragments were separated by agarose gel electrophoresis and analyzed by Southern blot using an oligonucleotide probe (5'GAATGGCTTATTCTATTGATAGC [Cortec #1778]) complementary to the 3' end of *flaI*. A 6 kb *Eco*RI digested fragment was identified (Figure 5). Restriction fragments were separated by gel electrophoresis and the mixture of digestion fragments in the area of the 6 kb *Eco*RI fragment were excised from the agarose and purified by the "Prep-A-Gene" method. The sticky ends of the digestion fragment were religated and inverse PCR amplification was performed using this circularized fragment as template and the primers 5'GAATGGCTTATTCTATTGATAGC (Cortec #1778) and 5'TTGCTTCTGGGAATCTAGTTCG (Cortec #1779). The annealing temperature and extension time for this reaction was 50°C and 8 min, respectively. Amplification products were not evident. The same reaction was performed using the Expand™ Long Template PCR System (Boerhinger Mannheim). Amplification products were not evident.

Using a different strategy, a restriction fragment encompassing *flaI* as well as downstream sequence was identified by Southern blot. *M. voltae* DNA was simultaneously digested with multiple restriction enzymes to find a digestion fragment of reasonable size for cloning into pUCBM21. A 2.2 kb *Eco*RI/*Hin*dIII double digest fragment was identified by agarose gel electrophoresis and Southern blot (Figure 6). The mixture of digestion fragments in the area of the 2.2 kb *Eco*RI/*Hin*dIII fragment were excised from the agarose, purified by the "Prep-A-Gene" method and ligated to *Eco*RI/*Hin*dIII digested pUC21. *E. coli* DH5α electrocompetent cells were then

Figure 5. Identification of *M. voltae* chromosomal DNA restriction fragments containing nucleotide sequence downstream of *flaI* by Southern blot (for the purpose of inverse PCR). *M. voltae* chromosomal DNA was digested with *Eco*RI (lane 1) or *Pst*I (lane 2), separated by agarose gel electrophoresis and probed with a DIG-labeled oligonucleotide (Cortec #1778). Markers are *Sty*I-digested λ DNA (lane M).

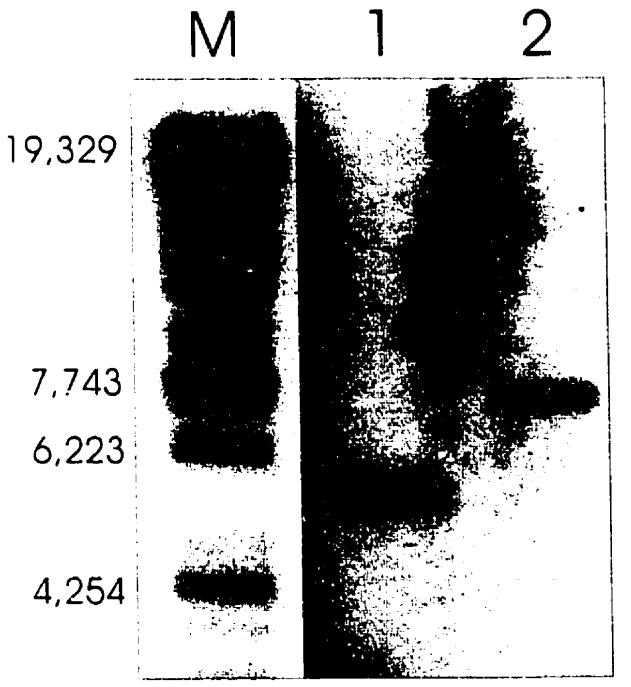
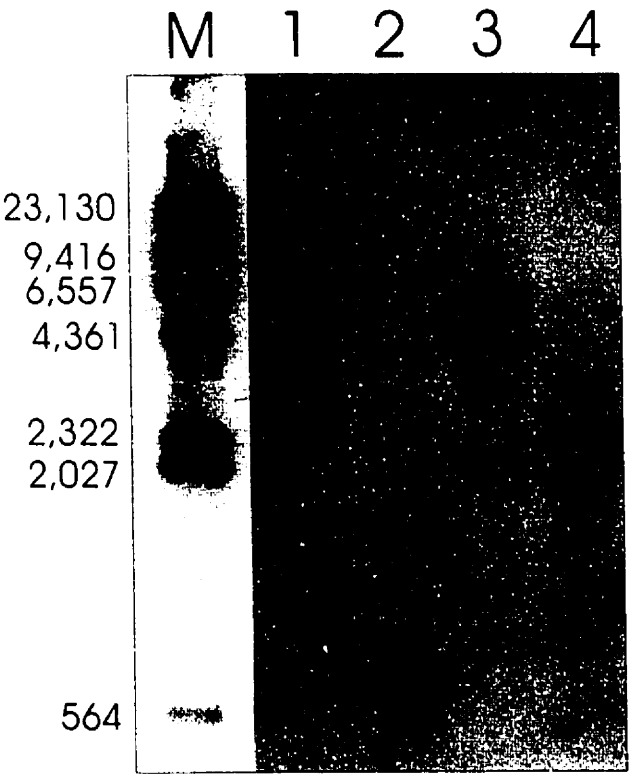

M
1
2
19,329
7,743
6,223
4,254

Figure 6. Identification of *M. voltae* chromosomal DNA restriction fragments containing nucleotide sequence downstream of *flaI* by Southern blot (for the purpose of cloning). *M. voltae* chromosomal DNA was digested with *Eco*RI (lane 1), *Eco*RI/*Eco*RV (lane 2), *Eco*RI/*Pst*I (lane 3), or *Eco*RI/*Hin*dIII (lane 4), separated by agarose gel electrophoresis and probed with a DIG-labeled oligonucleotide (Cortec #1778). Markers are *Hin*dIII-digested λ DNA (lane M).


M
1
2
3
4
23,130
9,416
6,557
4,361
2,322
2,027
564

transformed by electroporation and screened for pUCBM21 with insert by plating on LB medium supplemented with 100 μg/ml ampicillin and 40 μg/ml X-gal. Colonies containing the pUCBM21 vector (demonstrating the ampicillin resistance phenotype) were arbitrarily grouped and examined by colony hybridization using a DIG labelled oligonucleotide probe (Cortec #1778) to identify cells harbouring the pUCBM21 vector with the 2.2 kb insert of interest. These series of experiments were problematic because the pUCBM21/*flaJ* construct was positive for β-galactosidase activity (produced blue colonies on solid media supplemented with ampicillin and X-gal). Sequence not in frame with the *flaJ* gene does contain a start codon with a possible upstream ribosome-binding site that may be recognized by the *E. coli* polymerase. If translation starting from this site is in frame with the *lacZ* gene which is fused to the end of the 2.2 kb insert, it may have allowed LacZ to be produced, which would explain why constructs with insert produced blue colonies in *E. coli* cells grown on X-gal.

1.2. Sequencing analysis of *M. voltae flaJ* and protein comparison with other homolog(s)

Once identified, cells (designated KJ189) containing pUCBM21 with the *flaJ* insert (designated pJC1 or pKJ189) were cultured in LB medium and plasmid was purified by QIAGEN Midi Prep (Qiagen Inc.). pUC universal primers and unique primers (within the insert: 5'GAATGGCTTATTCTATTGATAGC [Cortec #1778] and 5'TGGGCGATTCTGTAAGTGC [Cortec #3646]) were used for the sequencing of the insert as conducted by the Cortec facility at Queens's University. A 1,677 nt gene which we have designated *flaJ* was identified (GenBank Accession No. AF068825; Figure 7).

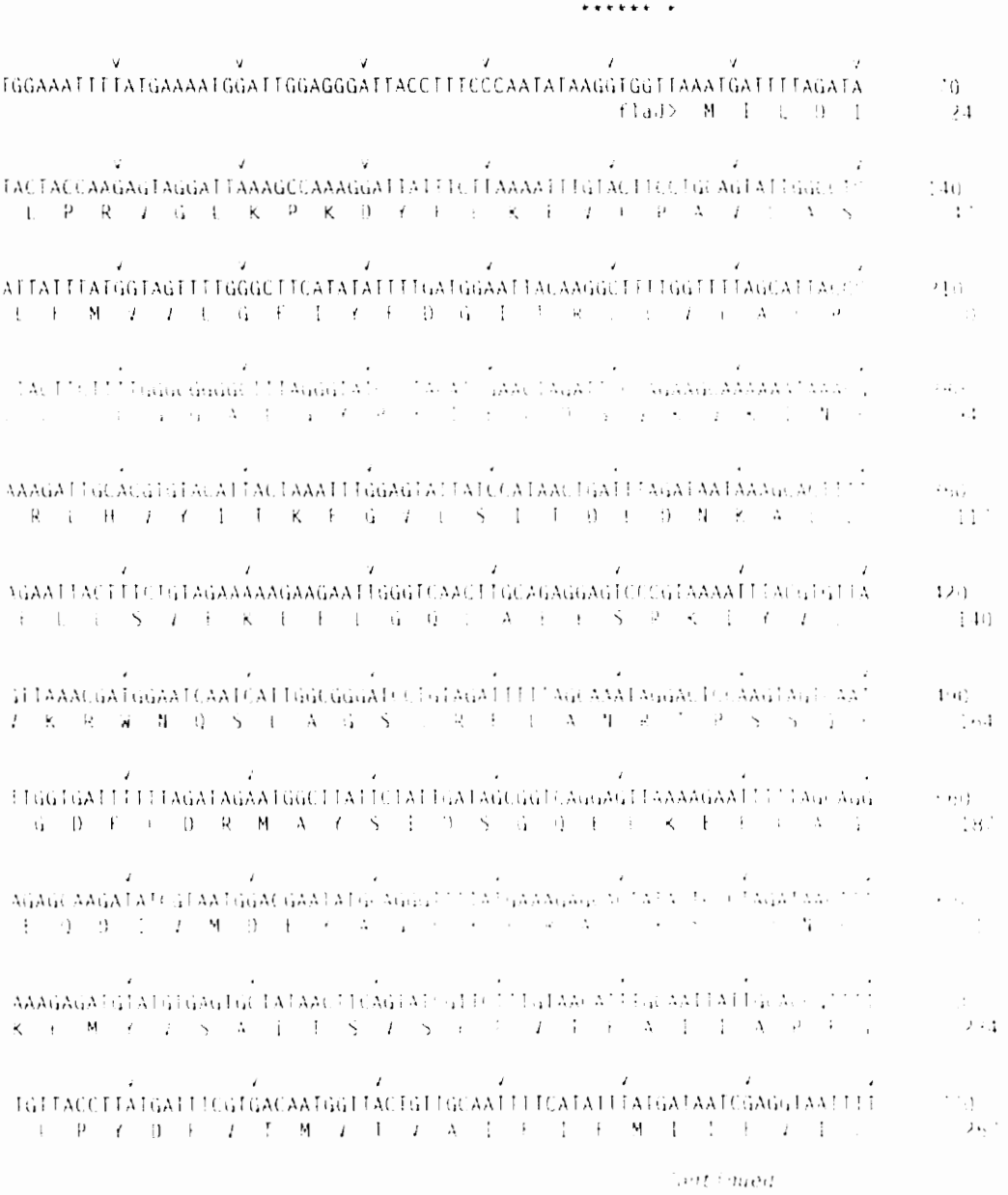


Figure 7. Nucleotide and deduced amino acid sequences of the putative flagella-related protein (FlaJ) of *M. voltae*. Coding region for *flaJ* (position 58 to 1734) is labelled. Asterisks (*) above the sequence represent a putative ribosome binding site. GenBank Accession numbers for the nucleotide and protein sequences are AF068825 and AAC19121, respectively.

A BLAST search (Altschul et al. 1990) of the available database revealed one highly related protein from each of *M. jannaschii* (ORF MJ0901; P ~ e^{-180}), *P. horikoshii* (ORF AP000002; P ~ e^{-87}), *P. abyssi* (ORF PAB1387; P ~ e^{-87}), *A. fulgidus* (ORF AE001030; P ~ e^{-19}) and *A. pernix* (ORF AP000062; P ~ e^{-17}) (Figure 8). A phylogenetic analysis of these proteins is presented in Figure 9.

Furthermore, nucleotide sequence obtained immediately downstream of *flaJ* indicated the presence of a gene homologous to the ORF MJ0973 in *M. jannaschii* (P ~ e^{-48}) (Figure 10), and not to the ORF MJ0902, but on the opposite strand. Since this gene was transcribed on the opposite strand to the flagella-related genes, *flaJ* appears to be the last gene in the flagella-related cluster in *M. voltae* (Figure 11). To further examine this postulate, a 500 bp stretch of *M. jannaschii* ORF MJ0902 (*flaK*) was amplified by PCR, DIG-labelled and used to probe *M. voltae* DNA for the presence, or absence, of a *flaK* equivalent (Figure 12). *M. jannaschii* and *M. voltae* *Eco*RI-digested DNA fragments and *Eco*RI/*Hin*dIII-digested pJC1 were separated by agarose gel electrophoresis, transferred to nitrocellulose membrane and hybridized with the DNA probe described above. A 15.5 kb restriction fragment, corresponding to *flaK* and additional flanking DNA sequence, was identified in *M. jannaschii*. Cross-reactive bands were not evident for *M. voltae* or pJC1.

flaJ encodes a 558 amino acid protein (Figure 7 and Table 6) with a predicted molecular weight of ~ 63 kDa and a theoretical pI of 8.31. Eight to nine transmembrane helices were identified using transmembrane predicting algorithms (TMpred) (Table 7) This is consistent with the presumed structure of the *M. jannaschii* equivalent (MJ0901) to *M. voltae* FlaJ.

```
M. voltae          ---MI----LDILPRVGLKPKDYFLKFVLPAVLAS--LFMVVLGFIYFDGITR---LLVL 48
M. jannaschii      MVIVV----FDLLPRVGLKPRDYLLRIVLPALITS--IVLILLGFMLFSGIIL---YIYL 51
P. horikoshii      MIPMPKEEKISIFVKADLNPKEYMKKILIPGLAGSGILFLVMGFFTRLITLPRGLVFFMY 60
P. abyssi          MIKVP-QEKISIFVKADLDPRTYLRRILLPGLLGSFVLFIVVSVFTRMIPLPRGLVFFMY 59
A. fulgidus        ----------------------MEVKTAKVPKLS-------LGFGRFG---------- 19
A. pernix          ----------------------MGLVLLGLPVAG--------GVALLMEPPT---SYLT 26

M. voltae          ALPLLLLGGALGYPYIELDSQKQKINERLHVYITKFGVLSITDLDNKALLELLSVEKEEL 108
M. jannaschii      LLPIIILVSAIGYPYIALDSQKNKINERLHIFITKFGTLSITDLNRKDLLKILSEEREEL 111
P. horikoshii      LVPAIIAVYVAAYPYLAADSKRLSINSKLPYFITYFAVLSTSEIGRTDILRVLAKD-PKL 119
P. abyssi          LIPIIIAIYVAAYPYLAADSKRISINSKLPYFITYFAVLSTSEIGRTDILKVLASD-PKL 118
A. fulgidus        ---KVLDVLRSRGKDLLMDN-------DLLFTLTYMASLSTANLSRDKIFEMVSEK--KE 67
A. pernix          AIVIMLTFAGLQAMLYLMASKIHVLDAELLYILLHMRLVASGKPPVGRIFRAIADFPQVY 86
                                                 *

M. voltae          GQLAEESRKIYVLVKRWNQSLAGSCRFLANRTPSSQFGDFLDRMAYSIDSGQELKEFLAG 168
M. jannaschii      GELAKESEKLYVLTDKWGRSLAEACRFLAQRTPSSEFADFLDRLAYALDSGEELKEFLIK 171
P. horikoshii      GAIANEMKKVYIIVDKLHRSLPEAFRFLAKRTPSKVFADFLDRLAYSLDSGVELKDYLFQ 179
P. abyssi          GAIAGEMKKVYMIVDKLHRSLPEAFRFLAKRTPSRVFADFLDRLAYSLDSGVELKDYLFQ 178
A. fulgidus        YAPSRYFSKVKDLTQKWHYDYATACDLIAEKIKHERLKKLFNRMANAIAAGEPDNEFLER 127
A. pernix          GGYSRLFRSIYDLGKEWGYSFPKAVSIIADSVEGEILRNILQRLSGVLAVGEDVEEFFER 146
                                                 *          *       *

M. voltae          EQDIVMDEYAGFYERALYSLDNFKEMYVSAITSVSFFVTFAIIAPFLLPY-DFVTMVTVA 227
M. jannaschii      EQDIVMDDYAAFYKRMLYSLDMYKELYVSAMTSIAFFLAFSILVPFLLPY-NFVFMATIA 230
P. horikoshii      EQQTVMDDYQTFYEGALYDLDIFKEIYESVIISIVFAGAFMIIGPILTGQ-NIGRLALYL 238
P. abyssi          EQQTVMDDYQTFYEGALYDLDIFKEIYESIIISIVFAGAFMIIGPIITGQ-NIGRLALYL 237
A. fulgidus        EWRAFKTIRKDEYERNLESLRKWSDAYTSLLVSASLISVVVLLSVIIYSAGDPGATLTAS 187
A. pernix          EYRTLLAEYENVYTRTMNSARVLLGIYVTMLGSLVFLVSTFMVLAFFFGG--DTRILYLS 204
                   *          *          *     *

M. voltae          IFIFMIIEVILIYSIKNKLPYDRLWHTGEKPTAIDRKLRKWLIISVGLTILASIVLFWGK 287
M. jannaschii      LFAFFAVELLIVVVIRNRLPFDRLWHTGEKPTETDIKLRKWLIISVILVVILLPFLLWAK 290
P. horikoshii      AFLILAAEIGSLLVIKYRMPEDPIWAEKRVETPRYRRIKRAAIISGMLVPIV--FLLYFA 296
P. abyssi          AFLILVAEIGSLMVIKYRMPEDPIWAEKRVETPRHRKIKRALIISSMLVPIV--FLLYFI 295
A. fulgidus        AFANFAISLFGVFMLFKAVPKDKKVHDLKIKSKEQTAISRLAPMLIPIAFFAVLFLTVLP 247
A. pernix          YAAVTVGAILLGLLVFMSLKQEPFEYRGNPEILRYKLLKAAGGLAIALGAAVG---AGII 261

                                                 Continued
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Figure 8. Amino acid sequence alignment of FlaJ proteins from *M. voltae*, *M. jannaschii*, *P. horikoshii*, *P. abyssi*, *A. fulgidus* and *A. pernix*. Identical amino acids are labelled with an asterisk (*). GenBank Accession numbers for the FlaJ homologs are Q58311 (*M. jannaschii*), BAA29648 (*P. horikoshii*), CAB50391 (*P. abyssi*), AAB90189 (*A. fulgidus*) and BAA80900 (*A. pernix*). This alignment was performed using the ClustalW WWW Service at the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw) (Thompson et al. 1994).

```
M. voltae          YIYEAP--QLLKIPYELIFSIAMTP----LMLGGYMAQREESLVIRKENNFPDFLRSLGD 341
M. jannaschii      YIVGLS--PFSQMPYMILVALGFTP----LAIGGFVALKEEEKVKRKEFVFPDFLRSLGD 344
P. horikoshii      LIR-----PRFHLPTPFVIALSLTP----LAYLGNVVRKEEGIIFRKDENFPAFIRSLAS 347
P. abyssi          LIR-----PRFKLPTPFVIALSLTP----LAYAGNVVRKEEAMIFRKDENFPAFIRSLAS 346
A. fulgidus        TLLNFTGGGLGILPKADYRGVGFLLAGLVLLPVGYLARIDDQKISKRDEAFTAFIRSLGA 307
A. pernix          YMRG----GLDFTGLVLAYAAAGLL----LLPVGVIARMEESKVRNVDEFFPVFIRSYGS 313
                                                 *   *                *  * **

M. voltae          SVSAKGGGTLESLGYLCTNDFGPLTKDLVALHRRLSIRINGQKSWKYFGHDTCSYLIQLF 401
M. jannaschii      SVSAKGGGMVSSLEYLSNHDFGPLTHDIKRLYKRLALGIDSNKSWRLFGFDSCSYLIQLF 404
P. horikoshii      SLAASGASLLLVLKYLSAHDFGMLTEDIRALYRRLAVRVDSARAWDFFIAETGSWLIGIF 407
P. abyssi          SLASSGASLLLVLKYLSAHDFGTLTEDIRSLYRRLAVRVDTVRAWDFFIAETGSWLIGIF 406
A. fulgidus        IKSGAGVSVAEALSRIDQKNLGELRELVMQLYRRLSMGLDAKLSWERFVGESGSYLISKL 367
A. pernix          HLSTVG-NMVKALEPLLISNLGILMGPIKRLYTRLKNSINPNVAWDLFSAETGSEMARRG 372
                        *       *         * *       *  **          *  *       *

M. voltae          SEMYERCTYLGGNSGQASHIIGKNFRKILRLRRSKYQNVNQFAGVMYGLSGGMALTLFAS 461
M. jannaschii      SDIFSRCIYFGGDPKTAAEIISKNFRKIVQLRKSKYQNIQQFVGVVYGLGGGLALALFAS 464
P. horikoshii      SEIFRESLRLGAEPDYVGKVISRNFERLVRLRRKRLQSVSNFIGIILGLTGAFAFSLAAS 467
P. abyssi          SEIFRESLRLGAEPDYVGKVISRNFERLVRLRRKRVQSVSNFIGIILGLTGAFAFSLAAS 466
A. fulgidus        TAIFVDATDLGGDADVVGEIVSSSNLEMVLLRLKRDLISSGFINLIIPLHLAMVGLVLFI 427
A. pernix          IIIFTDTVEAGGNPDLAGALISDHHNDMNRLRKLRYQVASTFSSTLFIMHGAAIIILLIM 432
                         *                        **          *

M. voltae          YGVASMVNGLYS--------------SLDIP-DTMLSMVHVVAPSDFGFISYMMYGTLI 505
M. jannaschii      LGVAKMINDLYS--------------SLSIP-ETVIHILNIAPISNVDVVEYIIFGSLI 508
P. horikoshii      FQVAVTINSIFS--------------KFQVPTEYIGNVIHVIPPSGLKLLDLSLIIIMI 512
P. abyssi          FQVAVAINDIFS--------------KFQVPTEYIGEIIHIIPPSGMKLLDLSLLLIMI 511
A. fulgidus        TQILAIFSDYISNLFASQLGGVSTGEVFSKIPGGMQGLNIGIFSGIPTDLLNQYSVWIIL 487
A. pernix          SKLLELFSNILQ--------------QLTTQLPPEIASIFPPFRSMDVSLLPILNLIFVT 477

M. voltae          IYALCSSYLIKLMDGGHYQVSLLHFSTMVWISSIVAVVTEMVTNSLLKAT---IPV---- 558
M. jannaschii      VYSIISAILIKIMDGGHKFVSLLHFVAILWICAIVAYITKLIVSQVLGVS---VPLY--- 562
P. horikoshii      VHSLLSALAIKVADGGNLLASLYYFVILLWVFAIGMYIGQVLMSKFMSVGGGSLILFLLG 572
P. abyssi          VHSLLSALAIKVADGGNLLGSLYYFVILLWVFAFGMYIGQTLMARFMSVGEGGMILHLLG 571
A. fulgidus        SLTIANTLAANVVKGGGRYLYLYYGAIFAILSGLLMLVVPPVVKWAFTLP---SFVE--- 541
A. pernix          TLVIVNSAVVTRVTPGSKTSFYFYLALYLLLSAGSIFVAVEIIDFVIGSIVVPQDIVPQL 537
                                 *

M. voltae          -
M. jannaschii      -
P. horikoshii      V 573
P. abyssi          V 572
A. fulgidus        -
A. pernix          -
```

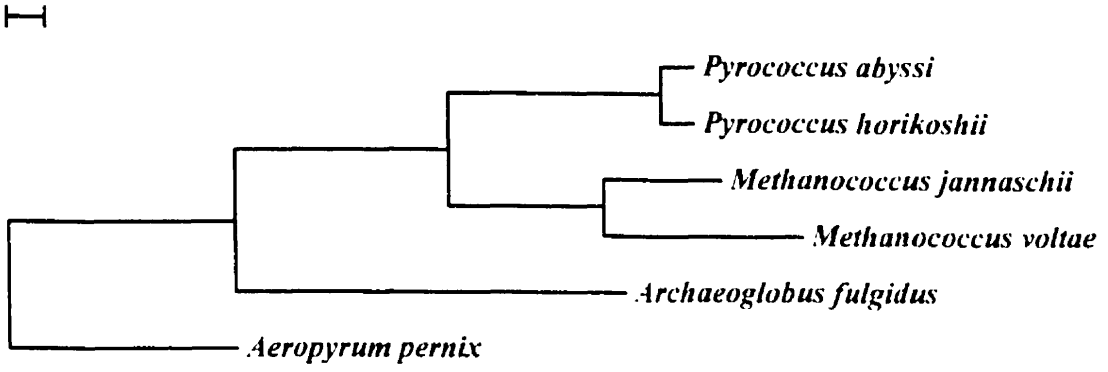
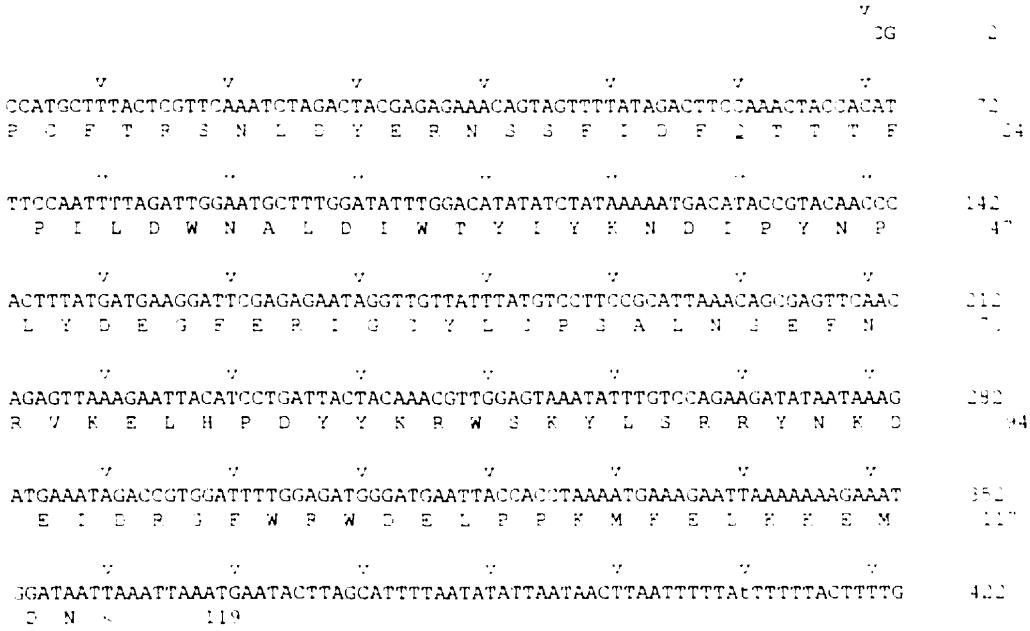


Figure 9 Phylogenetic tree of archaeal FlaJ proteins from *M. voltae*, *M. jannaschii*, *P. horikoshii*, *P. abyssi*, *A. fulgidus* and *A. pernix*. The bar represents a 10% amino acid sequence change. Note the grouping of the five FlaJ proteins from species of the Euryarchaeota kingdom.

Figure 10. Panel A. Nucleotide and deduced amino acid sequences of a putative protein-encoding ORF in *M. voltae* which is homologus to the ORF MJ0973 in *M. jannaschii*. Panel B. Schematic representation of *flaI* (orange arrow), *flaJ* (blue arrow) and the putative ORF (purple arrow) ending ~ 128 nt downstream of *flaJ*, but on the opposite strand.

A



B

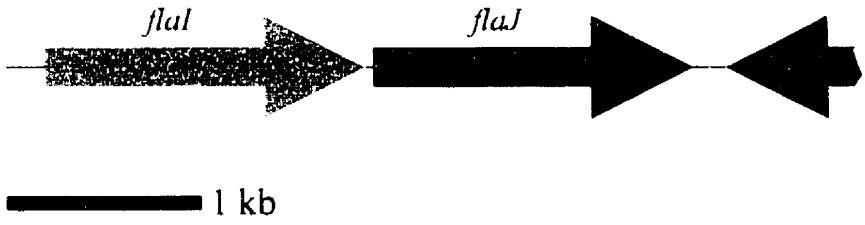


Figure 11. Schematic of the complete *M. voltae* flagellin gene region. The flagellin genes *flaA*, *flaB1*, *flaB2* and *flaB3* are based on Kalmokoff and Jarrell (1991). Seven additional genes were identified by Bayley and Jarrell (unpublished data) and were labeled *flaC* through *flaI*. This study has completed the sequence of the *M. voltae* flagellin gene region to the end of *flaJ*. Relative transcriptional start sites are indicated by "+1." The product of the *flaI* gene (orange) is homologous to PilT of the type IV pilus gene families.

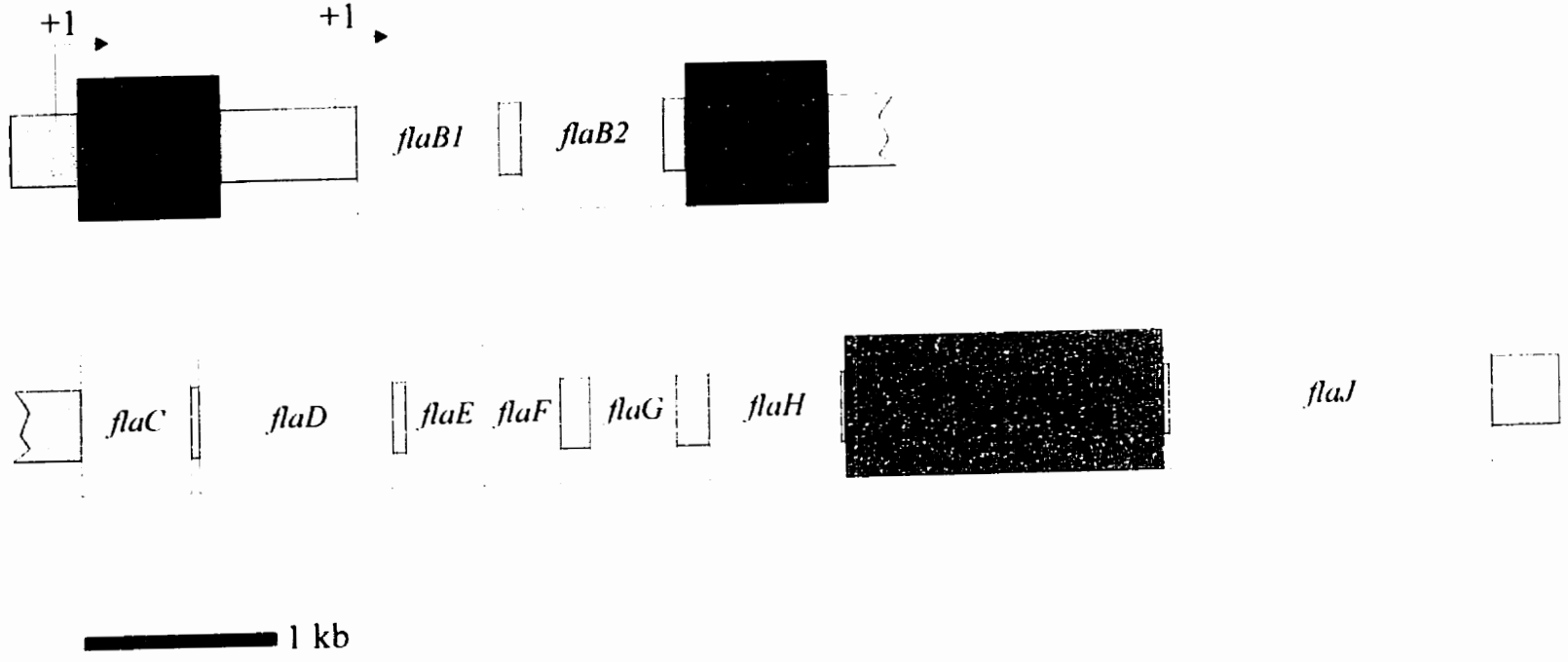

+1
+1
flaB1
flaB2
flaC
flaD
flaE
flaF
flaG
flaH
flaJ
1 kb

Figure 12. Identification of a *M. voltae* equivalent to *M. jannaschii flaK* by Southern blot. *Eco*RI-digested M. *jannaschii* (lane 1) and *M. voltae* (lane 2) chromosomal DNA and *Eco*RI/*Hin*dIII-digested pJC1 (lane 3) were separated by agarose gel electrophoresis and probed with a DIG-labeled 500 bp segment of *M. jannaschii flaK*. Markers are *Eco*RI/*Hin*dIII-digested λ DNA (lane M).

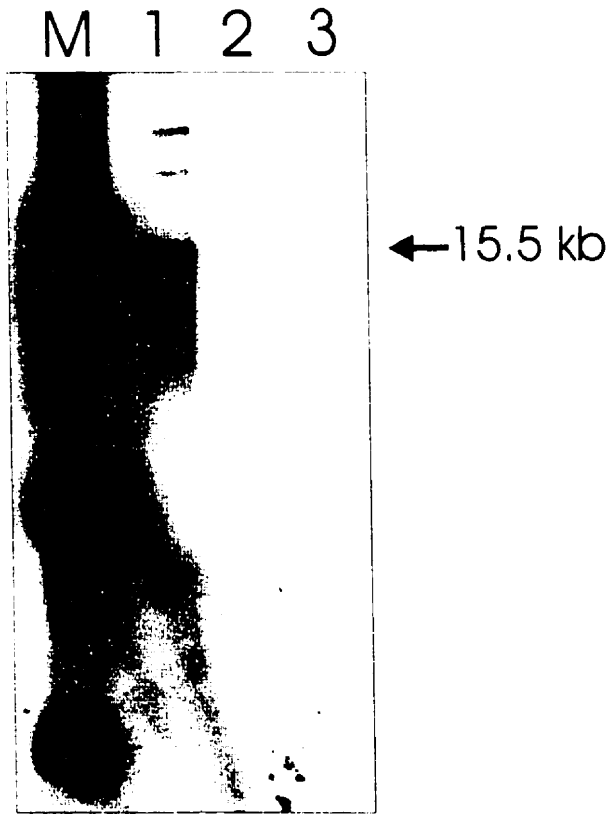

M 1 2 3
15.5 kb

Table 6. Amino acid composition of *M. voltae* FlaJ (558 aa).

Amino acid	Number of residues	Percentage
Ala (A)	32	5.7
Arg (R)	23	4.1
Asn (N)	16	2.9
Asp (D)	24	4.3
Cys (C)	5	0.9
Gln (Q)	15	2.7
Glu (E)	25	4.5
Gly (G)	40	7.2
His (H)	8	1.4
Ile (I)	45	8.1
Leu (L)	79	14.2
Lys (K)	29	5.2
Met (M)	21	3.8
Phe (F)	32	5.7
Pro (P)	18	3.2
Ser (S)	46	8.2
Thr (T)	24	4.3
Trp (W)	6	1.1
Tyr (Y)	32	5.7
Val (V)	38	6.8

Table 7. Suggested models for transmembrane topology*.

A. Strongly prefered model: N-terminus inside; 9 strong transmembrane helices, total score: 17,966

	From	To	Length	Score**	Orientation
1	21	39	19	2390	i-o
2	41	60	20	1766	o-i
3	199	216	18	2250	i-o
4	220	242	23	2683	o-i
5	268	286	19	2536	i-o
6	298	317	20	1372	o-i
7	446	468	23	1551	i-o
8	495	515	21	1945	o-i
9	530	548	19	1473	i-o

B. Alternative model; 8 strong transmembrane helices, total score: 15,823

	From	To	Length	Score**	Orientation
1	20	39	20	2491	o-i
2	41	60	20	1772	i-o
3	220	242	23	2683	o-i
4	268	286	19	2536	i-o
5	298	317	20	1372	o-i
6	446	468	23	1551	i-o
7	495	515	21	1945	o-i
8	530	548	19	1473	i-o

* prediction parameters: TM-helix length between 17 and 35
** only scores above 500 are considered significant

1.3. Expression of *M. voltae* FlaJ

One of the ongoing research objectives in the laboratory is the production of antibodies to flagellar-related gene products for use in localization and mutational studies. Consequently, the next logical step involved the expression of the *flaJ* gene product. Since expression systems in archaea are unavailable, bacterial expression technology was utilized. *E. coli* is the most frequently used prokaryotic expression system for high level production of heterologous proteins. *flaJ* was cloned into pET23a+ at *Nde*I and *Xho*I restriction sites (sites which are absent in *flaJ*). To do so, primers (5'GGAATTCCATATGATTTTAGATATACTACC [Cortec #3803] and 5'CCGCTCGAGAACTGGTATTGTAGC [Cortec #3804]) were designed to amplify *flaJ*, as well as add a 5' *Nde*I site (single underline) and a 3' *Xho*I site (double underline). The *Nde*I site created an ATG codon seven bases downstream of a ribosome binding site on the pET vector. In addition, the stop codon was deleted, creating an in frame fusion with a poly-histidine sequence corresponding to the C-terminal end of the protein. The amplified PCR product and the pET vector were then digested with *Nde*I and *Xho*I to expose their sticky ends and ligated to each other. pET23a+ containing *flaJ* was transformed into *E. coli* DH5α. Plasmid was isolated and transformed into *E. coli* BL21(DE3) and *E. coli* BL21(DE3) containing pLysS. Transformants were grown to an $O.D._{600}$ of 0.6 to 1 and induced with 0.4 mM IPTG. Total cell lysates of uninduced and induced cultures of *E. coli* BL21/pET23a+ and *E. coli* BL21/pET23a+/*flaJ* were analyzed by SDS-PAGE and stained with Coomassie Blue to identify induction products. Expression products were not evident. A plasmid stability test was performed with *E. coli* BL21(DE3)/pet23a+/*flaJ*. Colonies grew equally well on LB agar supplemented

with 1 mM IPTG, 1 mM IPTG and antibiotics, antibiotics alone or on LB agar with no IPTG or antibiotics. These results indicate that pET23a+ construct is carried, but the cells have lost the ability to express the target DNA and will grow in the presence of both antibiotics and IPTG. Induction of *E. coli* BL21(DE3)/pET23a+/*flaJ* was also performed at an incubation temperature of 28°C. However, expression products were still not evident. Cell density following induction was analyzed to determine whether culture growth decreased several hours after induction. This was not observed.

Total cell lysates of induced cultures (grown at 37°C) were purified under denaturing conditions (solubilized with 6 M urea) using Ni^{2+} affinity chromatography. The effluent was analyzed by SDS-PAGE and western blot using primary antibody specific to the polyhistidine sequence. Expression products containing the C-terminal His-tag were not evident.

Successful expression of proteins in a heterologous host is affected by a medley of variables, including impediments to transcription and translation elongation, mRNA stability and codon usage (Table 8). *E. coli*, as well as any other organism, displays a preference for certain codons. Consequently, heterologous genes that contain a substantial number of codons that are rarely used in *E. coli* may thus be expressed inefficiently. A second strategy involved the expression of FlaJ in the presence of a plasmid (pSJS1240) harboring and expressing the argU and ileX genes encoding rare tRNAs ($tRNA_{AGA\,AGG}$ and $tRNA_{AUA}$) (Kim et al. 1998). The genome of *M. voltae* has a G + C content of approximately 30%, hence the codon usage of genes from this organism is skewed favoring A or U at the third position. Consequently, expression of *M.*

Table 8. Codon usage in *E. coli*, *M. voltae* and *M. voltae flaJ*[a]

Amino Acid	Codon	*E. coli*	*M. voltae*	*flaJ*
Ala	GCA	20.4	33.2	35.8 (20)[b]
	GCC	25.2	3.5	1.8 (1)
	GCG	32.8	3.7	5.4 (3)
	GCU	16.1	32.8	14.3 (8)
Cys	UGC	6.4	5.1	0.0 (0)
	UGU	5.1	15.4	8.9 (5)
Asp	GAC	19.5	25.1	8.9 (5)
	GAU	32.1	37.3	34.0 (19)
Glu	GAA	39.9	67.9	28.6 (16)
	GAG	18.4	10.5	16.1 (9)
Phe	UUC	16.7	12.8	12.5 (7)
	UUU	21.7	18.8	44.7 (25)
Gly	GGA	8.3	22.4	23.3 (13)
	GGC	29.2	8.2	8.9 (5)
	GGG	11.0	4.8	8.9 (5)
	GGU	25.3	37.1	30.4 (17)
His	CAC	9.8	11.1	7.2 (4)
	CAU	12.6	6.0	7.2 (4)
Ile	AUA	4.9	23.9	44.7 (25)
	AUC	25.1	16.5	8.9 (5)
	AUU	29.8	35.8	26.8 (15)
Lys	AAA	34.4	67.4	41.1 (23)
	AAG	11.2	9.9	10.7 (6)
Leu	CUA	3.9	4.5	16.1 (9)
	CUC	10.7	4.1	3.6 (2)
	CUG	51.9	1.0	0.0 (0)
	CUU	11.1	12.7	21.5 (12)
	UUG	13.2	10.5	34.0 (19)
	UUA	13.4	47.7	66.2 (37)
Met	AUG	27.4	26.9	37.6 (21)
Asn	AAC	21.8	21.4	16.1 (9)
	AAU	18.0	27.3	12.5 (7)

[a]frequency per thousand
[b]number of codons

Table 8. Continued.

Amino Acid	Codon	*E. coli*	*M. voltae*	*flaJ*
Pro	CCA	8.4	19.1	8.9 (5)
	CCC	5.3	1.9	7.2 (4)
	CCG	22.8	2.5	7.2 (4)
	CCU	7.0	18.6	8.9 (5)
Gln	CAA	14.7	20.3	16.1 (9)
	CAG	29.0	5.4	10.7 (6)
Arg	AGA	2.5	24.7	23.3 (13)
	AGG	1.5	5.5	5.4 (3)
	CGA	3.6	1.7	3.6 (2)
	CGC	21.5	0.3	0.0 (0)
	CGG	5.5	0.6	0.0 (0)
	CGU	21.1	1.8	8.9 (5)
Ser	AGC	15.7	5.1	3.6 (2)
	AGU	8.8	11.1	16.1 (9)
	UCA	7.6	24.1	25.0 (14)
	UCC	8.9	4.0	12.5 (7)
	UCG	8.7	2.3	5.4 (3)
	UCU	9.1	9.1	19.7 (11)
Thr	ACA	7.5	24.8	10.7 (6)
	ACC	23.1	9.9	3.6 (2)
	ACG	14.0	4.2	3.6 (2)
	ACU	9.4	14.7	25.0 (14)
Val	GUA	11.2	31.5	37.6 (21)
	GUC	14.9	2.9	0.0 (0)
	GUG	25.8	5.8	8.9 (5)
	GUU	19.0	36.5	21.5 (12)
Trp	UGG	14.4	5.5	10.7 (6)
Tyr	UAC	12.4	19.0	26.8 (15)
	UAU	16.2	18.1	30.4 (17)
Ter	UAA	2.0	3.1	1.8 (1)
	UAG	0.3	0.3	0.0 (0)
	UGA	0.9	0.2	0.0 (0)

voltae proteins in *E. coli* can be problematic. pSJS1240 encodes two codon preferences for arginine and one for isoleucine which are very rare in *E. coli*. This method has been shown to be successful when overexpressing *M. jannaschii* proteins in *E. coli* (Kim et al. 1998), which otherwise were poorly expressed. *E. coli* BL21(DE3) containing pSJS1240 was transformed with the pET23a+/*flaJ* construct and expression was induced. Expression products were not evident by SDS-PAGE and western blot.

It has been demonstrated (Hannig and Makrides 1998) that the presence of rare codons near the 5' end of the transcript affects translational efficiency. A third strategy used to eliminate the negative effects of rare codons when overproducing a protein in a heterologous host was to induce production of an N-terminal truncated FlaJ. A forward primer (5'GGAATTCCATATGGCTTATTCTATTGATAGC [Cortec #5559]) was designed, and used with the reverse primer Cortec # 3804, to amplify a 1.2 kb segment of *flaJ* beginning at an ATG triplet 454 bp downstream of the original start codon and incorporating a 5' *Nde*I site (underlined). The codons immediately downstream of the new start codon were not one of the two codon preferences for arginine (AGG or AGA) or one for isoleucine (AUA) found commonly in *M. voltae* and very rare in *E. coli*. pET23a+ containing a 1.2 kb segment of *flaJ* was transformed into *E. coli* DH5α. Plasmid was isolated from these transformants and subsequently transformed into *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS. Expression products were not evident in total cell lysates analyzed by Coomassie Blue and His-tag western blot.

1.4. Transcriptional Analysis

To assess whether a *flaJ* transcript is produced after induction, total RNA isolated from *E. coli* BL21(DE3)/pET23a+, *E. coli* BL21(DE3)/pET23a+/*flaJ* and *E. coli*

BL21(DE3)/pET23a+/*flaJ*/pSJS1240 were analyzed by FA gel electrophoresis and northern hybridization using a DIG-labeled DNA probe (Cortec #1778). A 1.7 kb transcript, likely corresponding to *flaJ*, was identified in *E. coli* BL21(DE3)/pET23a+/*flaJ* and *E. coli* BL21(DE3)/pET23a+/*flaJ*/pSJS1240, but not in *E. coli* BL21(DE3)/pET23a+ (Figure 13). This suggests that mRNA was being produced in the *E. coli* strains containing the *flaJ* construct, and that the lack of protein production was likely related to problems arising during translation.

To assess whether a transcript containing *flaJ* could be detected from total RNA isolated from *M. voltae*, northern hybridization of *M. voltae* RNA was performed. A *flaJ* transcript was not clearly evident (Figure 13). Additional DNA probes were produced by PCR amplification of genomic DNA. The primer pairs used included Cortec #'s 3646/5466 and 6149/6150. These also gave no clear evidence of a *flaJ* transcript. A second approach utilized RT-PCR technology was employed. Amplification of *flaB1* was performed as a control for the experiment. A 650 bp amplification product was observed when RT-PCR was performed using DNase treated RNA and *flaB1* primers, as expected. No amplification products were evident when DNase treated RNA was run with primers (Cortec #1778 and #5466) designed to amplify a 630 bp segment of *flaJ* (Figure 14). *flaJ* is likely located on an unstable or low abundant transcript, consequently, mRNA with the *flaJ* gene is present at very low concentrations in total RNA isolated from *M. voltae*. In contrast, *flaB1* is present on a very abundant transcript.

Figure 13. Identification of transcripts containing *flaJ* in wild type *M. voltae* (lane 1) and *M. voltae* P-2 mutant (lane 2) total RNA and RNA isolated from *E. coli* BL21(DE3)/pET23a+ (lane 4 and 5), *E. coli* BL21(DE3)/pET23a+/*flaJ* (lane 6 and 7) and *E. coli* BL21(DE3)/pET23a+/*flaJ*/pSJS1240 (lane 8 and 9) by northern blot using a DIG-labeled oligonucleotide probe (Cortec #1778). Lanes 5, 7 and 9 contained twice the volumes of lanes 4, 6 and 8, respectively. Lane 3 was empty. Markers are indicated.

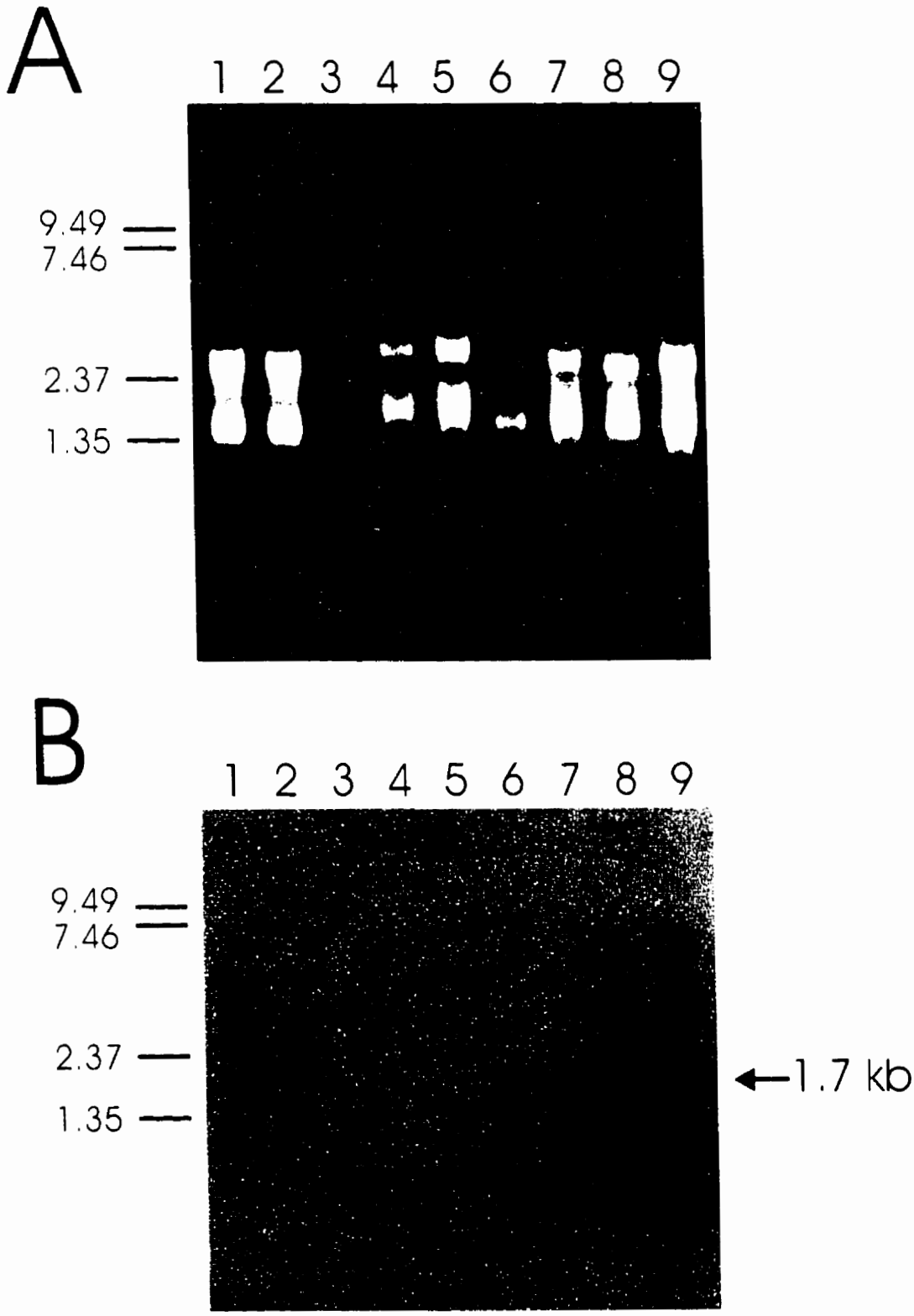
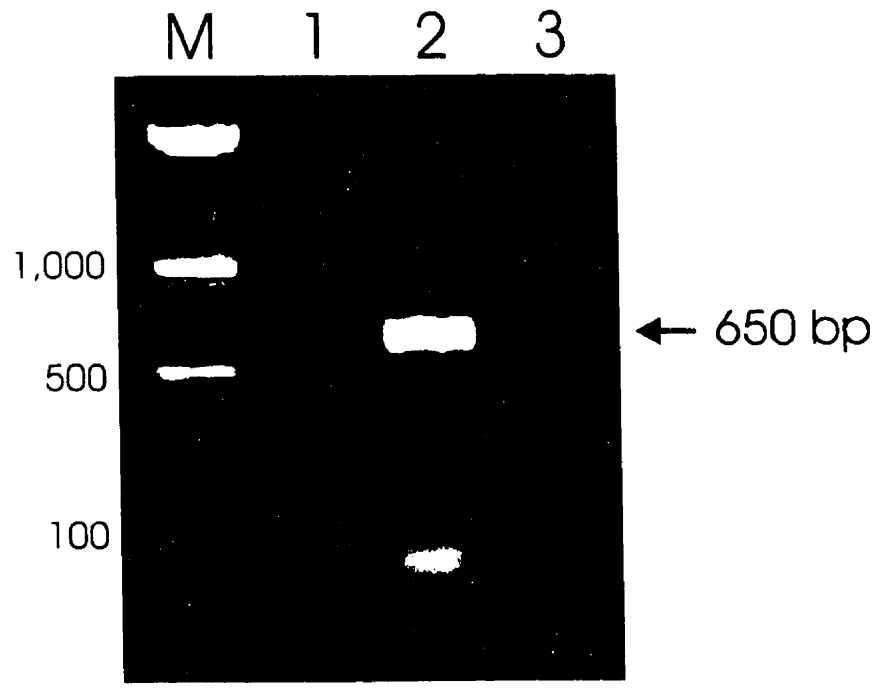

A
1 2 3 4 5 6 7 8 9
9.49
7.46
2.37
1.35
B
1 2 3 4 5 6 7 8 9
9.49
7.46
2.37
1.35
1.7 kb

Figure 14. Identification of transcripts containing *flaJ* in wild type *M. voltae* total RNA by reverse transcriptase PCR (RT-PCR). Total RNA was isolated from *M. voltae*, DNase treated (lane 2 and 3) and used as template in RT-PCR reactions with primers designed to amplify 650 bp or 630 bp segments of *flaB1* (lane 2) and *flaJ* (lane 3), respectively. Lane 1 contains RNase treated *M. voltae* RNA used as template in a RT-PCR reaction using primers designed to amplify *flaB1*. Markers used were a 100 bp ladder (lane M).


M
1
2
3
1,000
500
100
650 bp

2. Posttranslational processing of *M. voltae* preflagellin

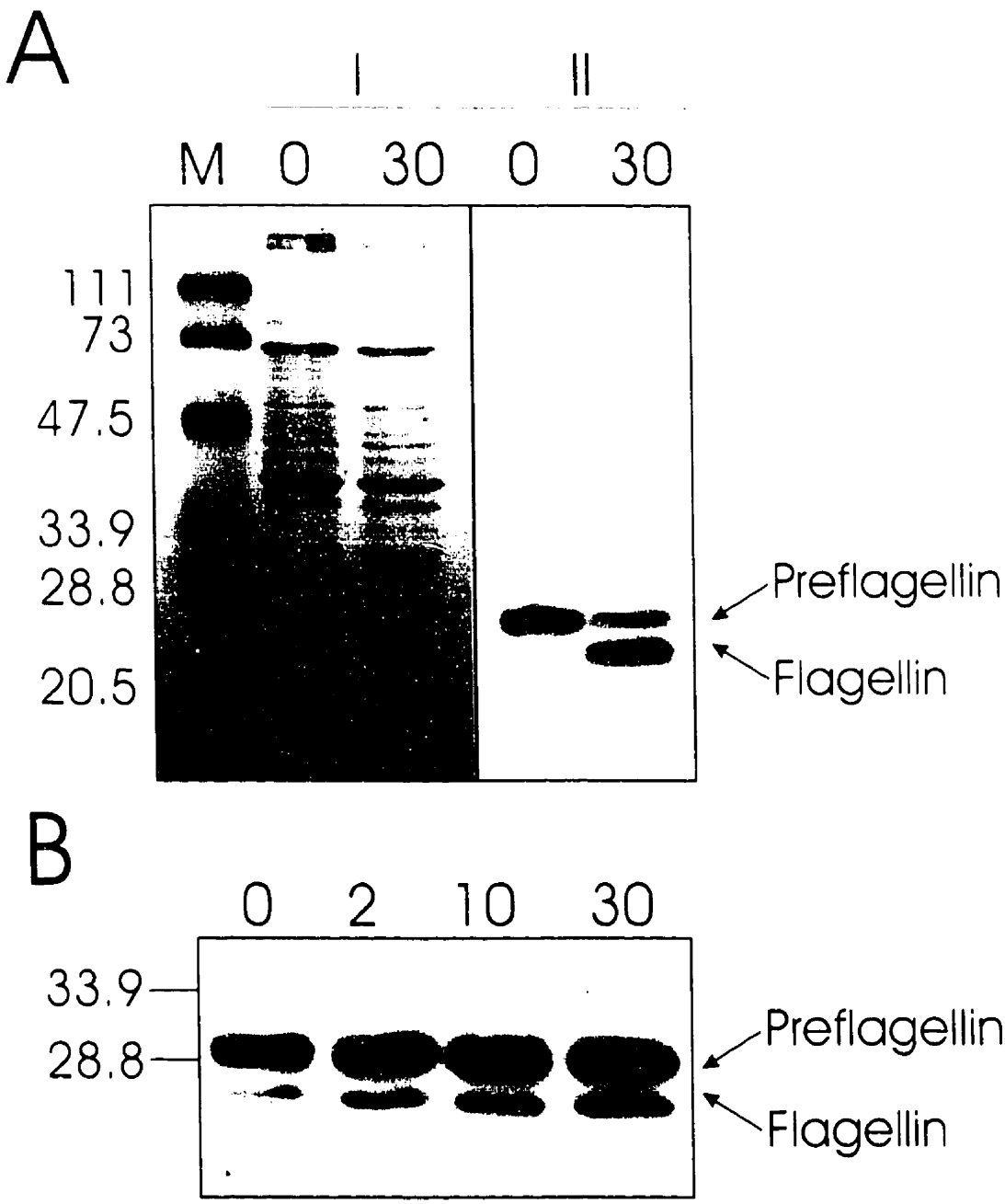
One of the unique features of archaeal flagellins is the presence of short leader peptides that are absent when examining mature subunits from isolated flagellar filaments. The presence of leader peptides on archaeal flagellins suggests that an enzymatic activity must be present in archaeal cells to process the preflagellins. Experiments to demonstrate a putative preflagellin peptidase activity was developed based on an *in vitro* prepilin peptidase assay system of *P. aeruginosa* (Strom et al. 1994)

2.1. Preflagellin peptidase activity of *M. voltae*

M. voltae FlaB2 (Bayley and Jarrell 1999) was expressed in *E. coli* using the pT7 system (Tabor and Richardson 1985). FlaB2 was detected in the crude *E. coli* membranes as a 26.5 kDa protein by both Coomassie staining and immunoblotting using antisera raised against the methanogen flagellin (Figure 15A). N-terminal analysis of the expressed protein revealed the first 10 amino acids to be MK1KEFMSNK which match exactly the predicted FlaB2 sequence with the attached leader peptide (Kalmokoff and Jarrell 1991).

In the case of FlaB1, *E. coli* BL21(DE3)/pLysS carrying pKJ202 were grown to log phase, induced, and analysed by SDS-PAGE to identify induction products. SDS-PAGE analysis revealed an induction product (with a C-terminal His-tag) from the strain carrying pKJ202 migrating at approximately 29 kDa detected by both Coomassie brilliant blue staining and immunoblotting with antisera raised to the FlaB2 flagellin of *M. voltae*. This induction product was detected only in *E. coli* harboring pET23a+ with *flaB1*. Also, this band was not detected when this strain was examined uninduced.

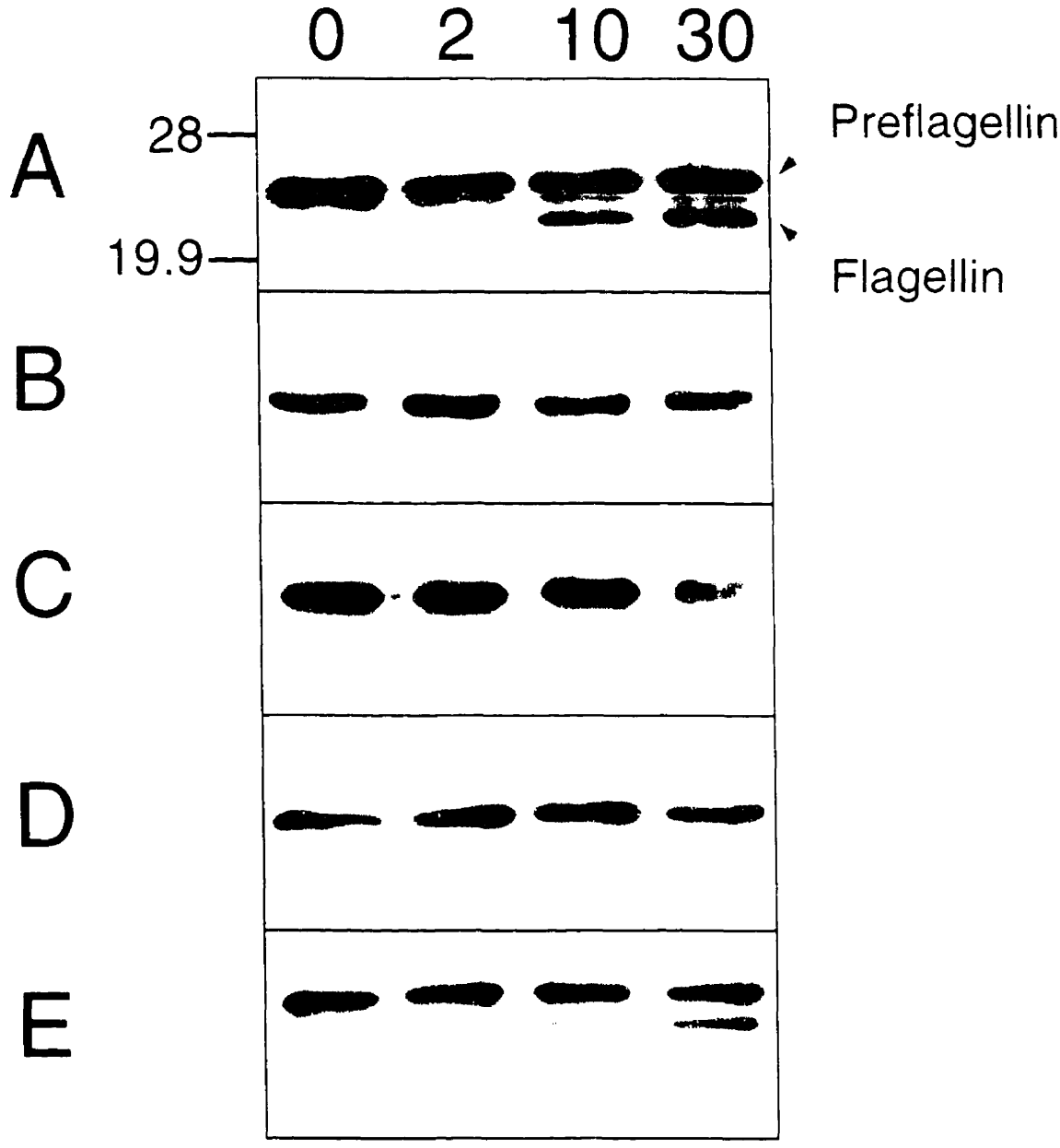
Figure 15. Processing of *M. voltae* preflagellins FlaB1 and FlaB2 by the *M. voltae* preflagellin peptidase. Panel A: the preflagellin peptidase reaction was performed with approximately 72 μg of induced *E. coli* KJ91 membranes (FlaB2 substrate source) combined with approximately 18 μg of *M. voltae* membranes (enzyme source) in 25 mM HEPES buffer (pH 8.5) containing 0.25% (vol/vol) Triton X-100 and 0.4 M KCl (optimized conditions), at a reaction temperature of 37°C. Ten μl samples were taken at 0 and 30 min time points and immediately mixed with 15 μl electrophoresis sample buffer and boiled for 5 min. Twenty and ten μl samples were analyzed by Coomasse Blue staining (I) and immunoblotting using a primary antibody dilution of 1:10,000 (II), respectively. The relative mobility of pre-stained SDS-PAGE low range molecular weight standards (Bio-Rad) are indicated in kDa (lane M). Panel B: the standard preflagellin peptidase reaction was performed with approximately 72 μg of induced *E. coli* KJ202 membranes (FlaB1 substrate source) combined with approximately 18 μg of *M. voltae* membranes (enzyme source) in 25 mM HEPES buffer (pH 7.5) containing 0.5% (vol/vol) Triton X-100. Samples removed at 0, 2, 10 and 30 min were similarly prepared and 10 μl aliquots were examined by immunoblotting using anti-FlaB2 antisera. Positions of 28 and 19.9 kDa molecular weight markers are indicated.


A
I
II
M
0
30
0
30
111
73
47.5
33.9
28.8
20.5
Preflagellin
Flagellin
B
0
2
10
30
33.9
28.8
Preflagellin
Flagellin

E. coli membranes were mixed with *M. voltae* envelopes in the presence of 25 mM HEPES buffer containing 0.5% (vol/vol) Triton X-100 and samples taken at various time points were analyzed by immunoblotting using anti-flagellin antisera. Western blot analysis of the preflagellin peptidase assay clearly demonstrated the appearance, with time, of a second cross-reactive band with greater electrophoretic mobility than the 26.5 kDa preflagellin (Figure 15A). This additional band increased in intensity with time and its size corresponded to that expected for the processed flagellin. The N-terminal sequence determined for this smaller (25 kDa), cross-reactive band was ASGIGT(L/G)IVF, indicating that it was indeed the product of the FlaB2 precursor after cleavage of its 12 amino acid N-terminal leader peptide. The 25 kDa flagellin was absent when the assay was performed without the addition of *M. voltae* membranes (Figure 16B) or with the addition of *M. voltae* membranes previously boiled for 5 min (Figure 16C). The addition of cardiolipin was not necessary for *M. voltae* preflagellin cleavage, unlike in the *in vitro* prepilin peptidase assay system of *P. aeruginosa* where an acidic phospholipid is an essential component (Strom et al. 1994). In the case of the *M. voltae* preflagellin peptidase, the appearance of the processed preflagellin proceeded equally well in the presence (Figure 16E) or the absence (Figure 16A) of cardiolipin. In addition, since all assays were conducted aerobically, the preflagellin peptidase did not require the strict anaerobic conditions that are essential for the growth of *M. voltae*.

E. coli membranes containing FlaB1 were similarly incubated with *M. voltae* membranes in the presence of 25 mM HEPES buffer containing 0.5% (vol/vol) Triton X-100 and the appearance of mature flagellin was observed by immunoblotting using anti-FlaB2 sera (Figure 15B). Although, both overexpressed FlaB2 and polyhistidine tagged

Figure 16. *In vitro* processing of *M. voltae* FlaB2 preflagellin is dependent on the addition of *M. voltae* membranes and detergent. The standard preflagellin peptidase reaction was performed with approximately 72 μg of induced *E. coli* KJ91 membranes (substrate source) combined with approximately 18 μg of *M. voltae* membranes (enzyme source) in 25 mM HEPES buffer (pH 7.5) containing 0.5% (vol/vol) Triton X-100 (A). The same reaction was performed without the addition of *M. voltae* membranes (B), with *M. voltae* membranes previously boiled for 5 min (C), without the addition of detergent (D) and with the addition of 0.05% (vol/vol) final concentration cardiolipin (E). Ten μl samples were removed at 0, 2, 10 and 30 min and then immediately mixed with 15 μl electrophoresis sample buffer and boiled for 5 min; 10 μl aliquots were examined by immunoblotting using a primary antibody dilution of 1:10,000. Positions of 28 and 19.9 kDa molecular weight markers are indicated.


0
2
10
30
28
19.9
Preflagellin
Flagellin
A
B
C
D
E

FlaB1 were suitable substrates of the preflagellin peptidase, the majority of the work described below utilized the FlaB2 substrate.

2.2. Conditions yielding maximal *M. voltae* FlaB2 preflagellin peptidase activity

Preflagellin cleavage activity absolutely required the addition of a buffer containing Triton X-100 or Nonidet P-40 (NP-40) as the solubilizing detergent. The processed flagellin was not evident in immunoblots when the assay was performed in reaction buffer that did not contain detergent (Figure 16D). Furthermore, preflagellin peptidase activity was not detected when Triton X-100 was replaced by a number of other nonionic detergents tested at a final concentration of 0.5% (vol/vol), including Tween 20, Tween 80, Brij 58, and SDS (Figure 17). Preflagellin cleavage was maximal near a final concentration of 0.25% (vol/vol) Triton X-100 as the solubilizing detergent. Concentrations of Triton X-100 higher than 0.5% (vol/vol) inhibited preflagellin cleavage (Figure 18A).

Since *M. voltae* is known to contain approximately 725 mM internal [K^+] (Jarrell et al. 1984), it is possible that if the preflagellin peptidase has its active site on the inner face of the cytoplasmic membrane (as observed for the prepilin peptidase (Lory 1994)), its activity could be greatly influenced by the final concentration of K^+ in the assay buffer. To determine whether the addition of K^+ would affect the efficiency of preflagellin cleavage, the standard reaction buffer, which contains no added K^+, was supplemented with KCl to obtain a final concentration between 0.2 and 1.2 M. Although peptidase activity was evident in the absence of salt, cleavage activity increased with the addition of KCl, with maximal cleavage observed in buffer containing 400 mM KCl

Figure 17. *In vitro* processing of *M. voltae* FlaB2 preflagellin by the *M. voltae* preflagellin peptidase and the requirement of a solubilizing detergent. The standard preflagellin peptidase reaction was performed with approximately 72 μg of induced *E. coli* KJ91 membranes (substrate source) combined with approximately 18 μg of *M. voltae* membranes (enzyme source) in 25 mM HEPES buffer (pH 7.5) containing 0.5% (vol/vol) Triton X-100 (A), Nonidet P-40 (B), Tween 20 (C), Tween 80 (D), Brij 58 (E) or sodium dodecyl sulphate (F). Ten μl samples were removed at 0, 2, 10 and 30 min and then immediately mixed with 15 μl electrophoresis sample buffer and boiled for 5 min; 10 μl aliquots were examined by immunoblotting using a primary antibody dilution of 1:10,000. Positions of 28 and 19.9 kDa molecular weight markers are indicated.

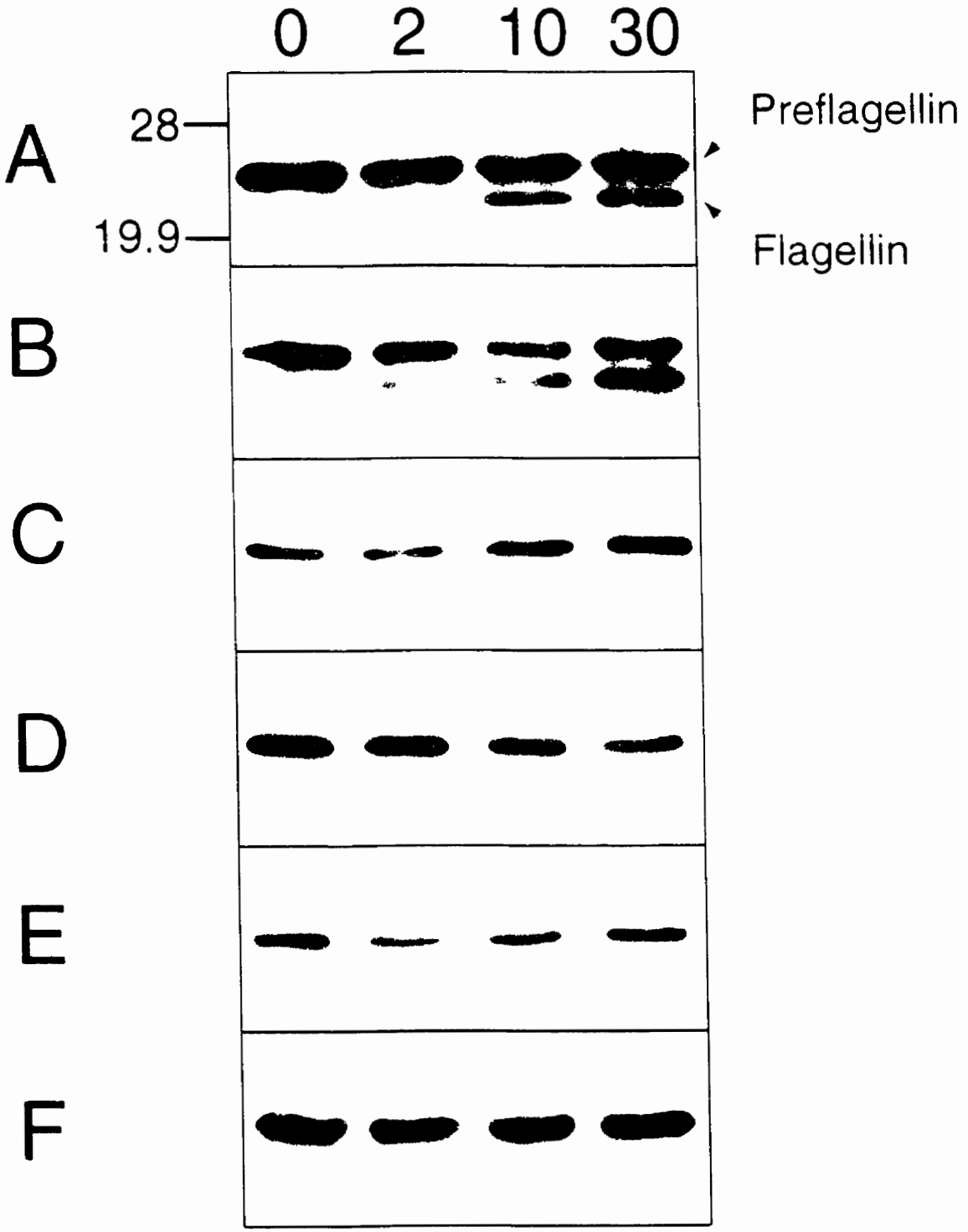
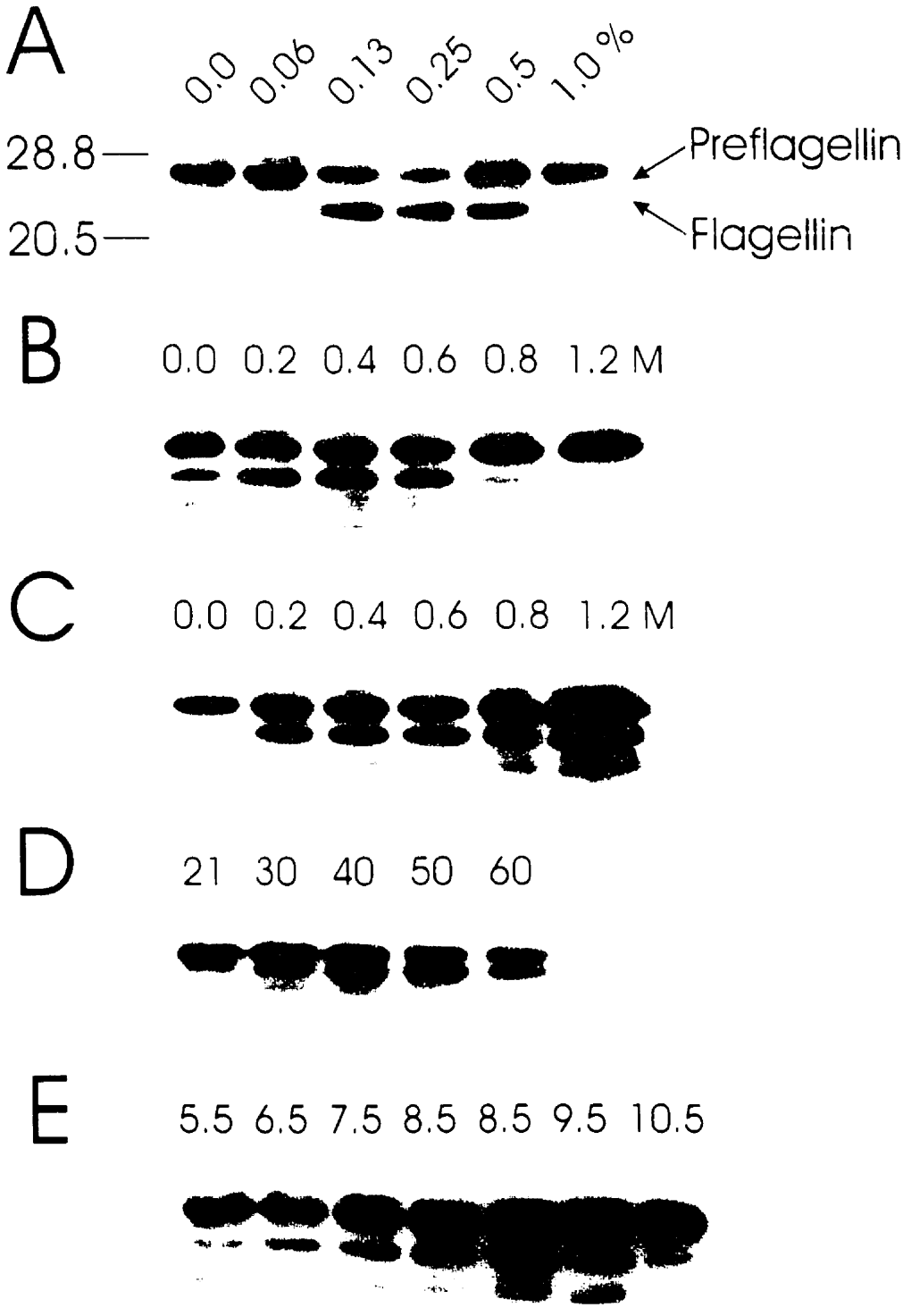

0
2
10
30
28
19.9
A
B
C
D
E
F
Preflagellin
Flagellin

Figure 18. Determination of detergent, salt, temperature and pH conditions yielding maximum preflagellin peptidase activity. For all reactions, 10 µl samples were removed at 10 min and then immediately mixed with 15 µl electrophoresis sample buffer and boiled for 5 min; 10 µl aliquots were examined by immunoblotting using a primary antibody dilution of 1:10,000. Panel A: the standard preflagellin peptidase reaction was performed with approximately 72 µg of induced *E. coli* KJ91 membranes (substrate source) combined with approximately 18 µg of *M. voltae* membranes (enzyme source) in 25 mM HEPES buffer (pH 7.5) containing varying final concentrations of Triton X-100 (0.0, 0.0625, 0.125, 0.25, 0.5 and 1.0% [vol/vol]). Panel B: the standard preflagellin peptidase reaction (25 mM HEPES [pH 7.5] containing 0.5% [vol/vol] Triton X-100) was supplemented with KCl to a final concentration of 0.0, 0.2, 0.4, 0.6, 0.8 or 1.2 M. Panel C: the standard preflagellin peptidase reaction was supplemented with NaCl to a final concentration ranging from 0.0, 0.2, 0.4, 0.6, 0.8 or 1.2 M. Panel D: the standard preflagellin peptidase reaction was performed at reaction temperatures of 21, 30, 40, 50 and 60°C. Panel E: the standard preflagellin peptidase reaction was performed in 25 mM MES buffer (pH 5.5, 6.5), 25 mM HEPES buffer (pH 7.5, 8.5) and 25 mM Bis Tris Propane buffer (pH 8.5, 9.5, 10.5).


A
0.0
0.06
0.13
0.25
0.5
1.0 %
28.8
20.5
Preflagellin
Flagellin
B
0.0 0.2 0.4 0.6 0.8 1.2 M
C
0.0 0.2 0.4 0.6 0.8 1.2 M
D
21 30 40 50 60
E
5.5 6.5 7.5 8.5 8.5 9.5 10.5

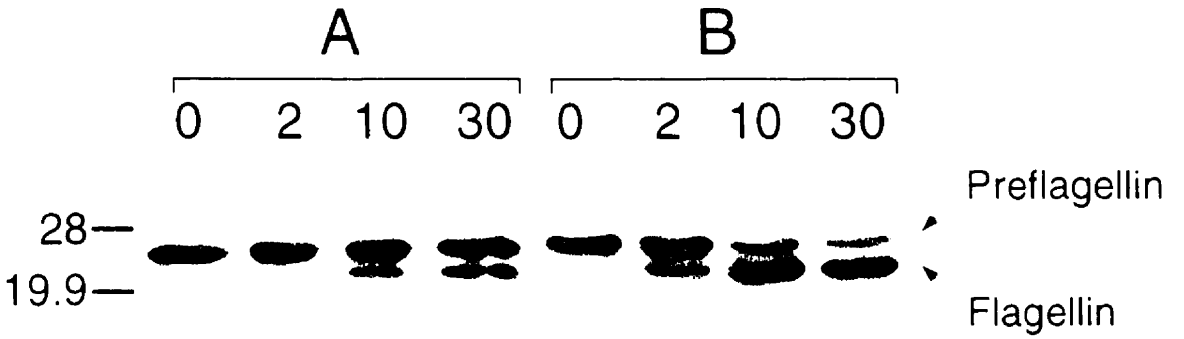
Addition of KCl above 400 mM resulted in inhibition of the preflagellin cleavage with no preflagellin peptidase activity detected in the presence of 1.2 M KCl (Figure 18B).

M. voltae is a moderate halophile, growing over a wide range of NaCl concentrations but with a reported optimum of 0.4 M (Whitman et al. 1982). Thus, if the preflagellin peptidase has an active site on the outer surface of the cytoplasmic membrane as does leader peptidase I (Dalbey 1991), then it is possible its activity could be greatly influenced with the addition of NaCl to the reaction buffer. Preflagellin cleavage activity increased with the addition of NaCl to 0.2 M, but was not markedly influenced by the further addition of NaCl to 1.2 M final concentration (Figure 18C).

M. voltae grows optimally at pH 6.0 to 7.0 and 35 to 45°C (Whitman et al. 1982). *In vitro* peptidase activity was apparent across a broad pH range of 6.5 to 9.5 and was maximal at approximately pH 8.5. No activity was observed at pH 5.5 or pH 10.5 (Figure 18E). Peptidase activity was maximal near 40°C with activity detected over the temperature range from 30 to 60°C after an incubation time of 10 min. No activity was detected after a 10 min reaction time when the assay was performed at room temperature (21°C; Figure 18D).

A comparison of the initial assay conditions based on the prepilin peptidase system [25 mM Hepes buffer pH 7.5 containing 0.5% (vol/vol) Triton X-100, 37°C] with the amended assay conditions developed in this work [25 mM Hepes buffer pH 8.5 containing 400 mM KCl and 0.25% (vol/vol) Triton X-100, 37°C] is presented in Figure 19. Under amended conditions, the processed form of the flagellin becomes the predominant of the two flagellin species over the 30 min time course of the reaction.

Figure 19. Comparison of the standard and amended reaction conditions for the *in vitro* processing of *M. voltae* FlaB2 preflagellin by the *M. voltae* preflagellin peptidase. The standard preflagellin peptidase reaction (A) performed with approximately 72 μg of induced *E. coli* KJ91 membranes (substrate source) combined with approximately 18 μg of *M. voltae* membranes (enzyme source) in 25 mM HEPES buffer (pH 7.5) containing 0.5% (vol/vol) Triton X-100 and the amended preflagellin peptidase reaction (B) performed in 25 mM HEPES buffer (pH 8.5) containing 0.4 M KCl and 0.25% (vol/vol) Triton X-100. Ten μl samples were removed at 0, 2, 10 and 30 min and then immediately mixed with 15 μl electrophoresis sample buffer and boiled for 5 min. 10 μl aliquots were examined by immunoblotting using a primary antibody dilution of 1:10,000. Positions of 28 and 19.9 kDa molecular weight markers are indicated.


A
B
0
2
10
30
0
2
10
30
Preflagellin
28—
19.9—
Flagellin

A non-flagellated *M. voltae* mutant (*M. voltae* P-2 [Jarrell et al. 1996a]) was also examined for preflagellin peptidase activity under the standard reaction conditions. This mutant has an insertional vector located in *flaB2*, which also results in a polar effect on the co-transcribed downstream genes. Preflagellin peptidase activity using *M. voltae* P-2 membranes was comparable to that observed in the membranes of wildtype *M. voltae* cells after a reaction time of 30 min (Figure 20).

2.3. Heterologous preflagellin peptidase activity

All species of the genus *Methanococcus* tested (*M. deltae*, *M. maripaludis*, *M. vannielii*, *M. voltae*, *M. thermolithotrophicus* and *M. jannaschii*) exhibited preflagellin peptidase activity against FlaB2 of *M. voltae* expressed in *E. coli*, with the sole exception of the hyperthermophile *M. igneus* (Figure 21). Interestingly, it is unclear whether the few filamentous structures observed on the surface of *M. igneus* are, in fact, flagella (Burggraf et al. 1990). If *M. igneus* truly does lack flagella, its lack of preflagellin peptidase activity is readily explained.

M. thermolithotrophicus demonstrated excellent preflagellin peptidase activity at 60°C (Figure 21) as well as 37°C (data not shown). Unexpectedly, preflagellin peptidase activity of *M. jannaschii* was observed at 60°C, but not at 80°C, close to its optimum growth temperature of 85°C. Since *M. jannaschii* is flagellated at 80°C and thus would be expected to have an active preflagellin peptidase at this temperature, we surmised the absence of preflagellin peptidase activity at 80°C may be due to the instability of the *M. voltae* substrate at this elevated temperature. FlaB2 stability was examined by preheating *E. coli* KJ91 membranes containing FlaB2 for 10 minutes at 60°C or 80°C and

Figure 20. *In vitro* processing of the FlaB2 preflagellin by *M. voltae* and the non-flagellated *M. voltae* mutant (*M. voltae* P-2). The standard preflagellin peptidase reaction was performed with approximately 72 μg of induced *E. coli* KJ91 membranes (substrate source) combined with approximately 18 μg of either *M. voltae* (A) or *M. voltae* P-2 membranes (B) (enzyme source) in 25 mM HEPES buffer (pH 7.5) containing 0.5% (vol/vol) Triton X-100. Ten μl samples were removed at 0, 2, 10 and 30 min and then immediately mixed with 15 μl electrophoresis sample buffer and boiled for 5 min; 10 μl aliquots were examined by immunoblotting using a primary antibody dilution of 1:10,000. Positions of 28 and 19.9 kDa molecular weight markers are indicated.

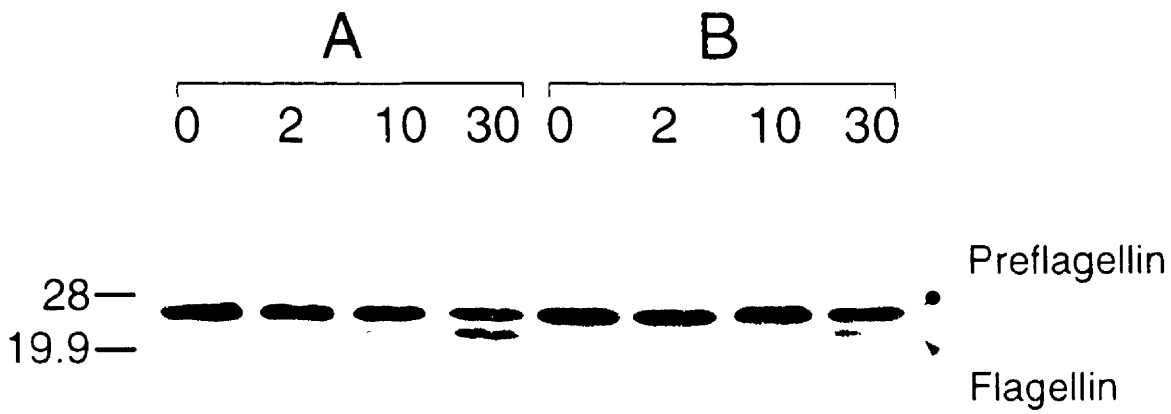
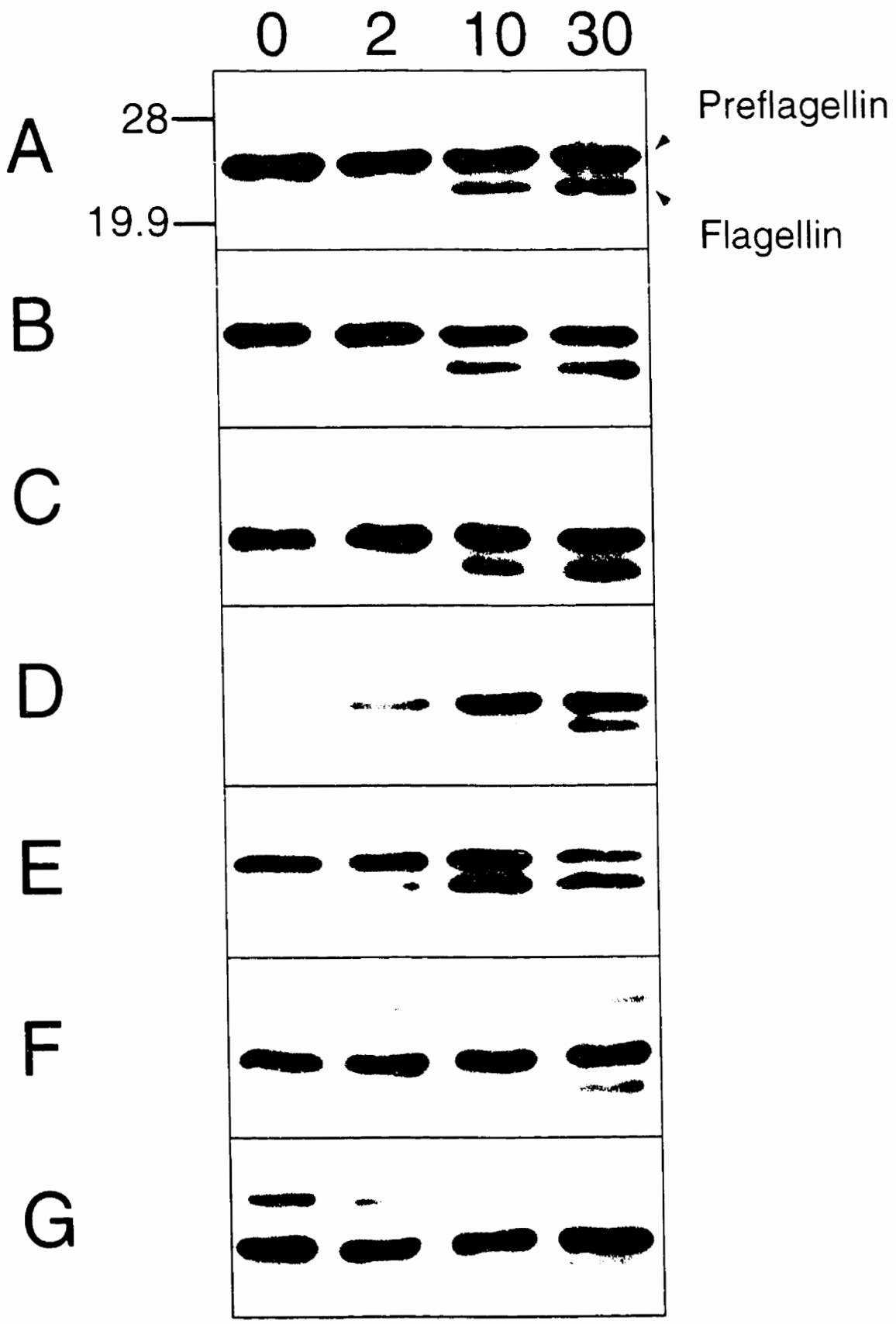

A
B
0
2
10
30
0
2
10
30
28—
19.9—
Preflagellin
Flagellin

Figure 21. *In vitro* processing of *M. voltae* FlaB2 preflagellin by the preflagellin peptidase of *M. voltae* and other methanococci. The standard preflagellin peptidase reaction performed with approximately 72 μg of induced *E. coli* KJ91 membranes (substrate source) combined with approximately 18 μg of *M. voltae* membranes (A) in 25 mM HEPES buffer (pH 7.5) containing 0.5% (vol/vol) Triton X-100, performed at a reaction temperature of 37°C. The same reaction was performed using *M. deltae* membranes (B), *M. maripaludis* membranes (C) or *M. vannielii* membranes (D) in place of *M. voltae* membranes. The same reaction was performed at a reaction temperature of 60°C using *M. thermolithotrophicus* membranes (E), *M. jannaschii* membranes (F) or *M. igneus* membranes (G) as the enzyme source. Ten μl samples were removed at 0, 2, 10 and 30 min and then immediately mixed with 15 μl electrophoresis sample buffer and boiled for 5 min; 10 μl aliquots were examined by immunoblotting using a primary antibody dilution of 1:10,000. Positions of 28 and 19.9 kDa molecular weight markers are indicated.


0
2
10
30
28
19.9
Preflagellin
Flagellin
A
B
C
D
E
F
G

subsequently performing an assay for preflagellin peptidase activity using *M. voltae* membranes at a reaction temperature of 37°C (data not shown). Peptidase activity was unaffected by the pretreatment at either temperature.

We also tested other flagellated methanogens with a similar wall profile to the *Methanococcus* species for the presence of a preflagellin peptidase active against *M. voltae* FlaB2. Membranes isolated from *M. marisnigri* and *M. cariaci* did not cleave the FlaB2 preflagellin of *M. voltae* under the standard reaction conditions (data not shown). No attempt to detect preflagellin peptidase activity in these methanogens by altering the standard reaction conditions was done.

Chapter 4. Discussion

The principle objective of this study was the characterization of a putative preflagellin peptidase activity in the mesophilic archaeon *M. voltae*, an action likely analogous to the one performed by the prepilin peptidase of the type IV pilus system. Preliminary data on the characterization of the non-flagellated mutant *M. voltae* P-2 (Jarrell et al. 1996a) suggested that a putative preflagellin peptidase was located on the polycistronic transcript encoding the FlaB flagellins. Previously, sequencing of the flagellin gene region in *M. voltae* was completed to the end of *flaI* (Bayley and Jarrell, unpublished data). In this study, the *flaJ* gene from *M. voltae* was cloned using the *flaI* gene as a probe for several Southern hybridizations. This gene represents the last ORF in the flagellin gene region of *M. voltae*. The putative product of this gene is homologous to hypothetical proteins from a number of flagellated organisms, including the related methanogen *M. jannaschii* and the Archaea *P. horikoshii*, *P. abyssi*, *A. fulgidus* and *A. pernix*. The function of this putative gene product is as yet unknown. However, a number of possible transmembrane domains may indicate a membranous destination *flaJ* likely encodes an archaeal-specific flagella accessory protein which may provide a function similar to those provided by the ancillary gene products of the type IV pilus system (e.g., peptidase or pore). Expression of the *flaJ* gene product in *E. coli* was unsuccessful using the pET system and may not be possible with current expression technology. Although significant progress in many technical aspects of gene expression in *E. coli* have been made, the problems associated with the expression of archaeal membrane proteins in *E. coli* have yet to be addressed.

Recent research on archaeal flagellins has unveiled a number of interesting traits, such as the presence of short leader peptides and their sequence similarity to type IV pilins and not bacterial flagellins (Jarrell et al. 1996b). This study has identified preflagellin peptidase activity in the membranes of several, flagellated methanococci, including mesophiles, a thermophile and a hyperthermophile. Preflagellin peptidase activity was confirmed by comparison of the N-terminal sequences of the expressed preflagellin and the processed flagellin. This is the first report of such an enzymatic activity. Preflagellin cleavage activity was found in the membrane fraction of *M. voltae* and is maximal (for the conditions tested) near 40°C, pH 8.5 in the presence of 0.4 M KCl with 0.25% (vol/vol) Triton X-100 as the solubilizing detergent. The prepilin peptidase assay devised by Strom et al. (1994) required the addition of cardiolipin, an acidic phospholipid likely required to improve the formation of lipid micelles. In contrast, our assay was equally effective in the absence of cardiolipin. It should be noted, however, that Strom et al. (1994) combined two different bacterial membranes, whereas, our assay system required the mixture of biochemically dissimilar bacterial and archaeal membranes. Direct demonstration of preflagellin processing adds more evidence to the description of archaeal flagellation as a unique motility structure, distinct from that of its bacterial counterpart and with several similarities to type IV pili. Since searches of the complete genome sequences reported for flagellated archaea do not reveal homologs to prepilin peptidases, archaeal preflagellin peptidases may represent a new class of proteolytic enzymes.

The active site of leader peptidase I is located on the periplasmic side of the cytoplasmic membrane (Dalbey 1991) while that for the prepilin peptidase faces the

cytoplasm (Lory 1994). When we analyzed the cation dependence of the preflagellin peptidase activity, a significant difference in the response to varying K^+ and Na^+ concentrations was observed. Preflagellin processing was optimal at approximately 0.4 M KCl, with no activity observed at the higher concentrations tested (0.8 to 1.2 M). On the other hand, preflagellin processing was evident over a wide [Na^+] range, with excellent activity observed even at 1.2 M NaCl, well above the [K^+] that totally inhibited the processing. The results obtained with KCl suggest that if a preflagellin peptidase has its active site located on the cytoplasmic side of the cytoplasmic membrane then its activity would be well below optimal given that the internal [K+] concentration is 0.725 M (Jarrell et al. 1984). The high level of activity, observed throughout a broad range of [NaCl], including the optimal [NaCl] for growth (0.4 M [Whitman et al. 1982]), indicates that near optimal activity would be exhibited by the preflagellin peptidase if its active site were located on the periplasmic side of the cytoplasmic membrane. While these results might suggest an active site on the periplasmic face of the cytoplasmic membrane, since many enzymes do not function optimally at the salt concentration in which they are normally found, it would be premature to draw definitive conclusions on the orientation of the active site of the preflagellin peptidase based solely on these results.

Cleavage of the preflagellin leader peptide occurs following an invariant glycine residue in *M. voltae* and *M. vannielii* and likely in all other archaeal preflagellins (Table 9). The –2 and –3 positions in archaeal flagellins are always held by charged amino acids, usually a basic amino acid (lysine or arginine) but in the case of *H. salinarum* glutamic acid is found (Table 9). This is very different from the situation of leader peptidase I where cleavage requires small uncharged amino acids at positions –1 and –3

Table 9. N-terminal sequences of archaeal preflagellins*.

Precursor Protein	GenBank accession no.	N-terminal sequence (arrow indicates demonstrated or predicted cleavage site)
M. voltae		
FlaA	P27802	MKVKEFMNNKKG↓ATGVG
FlaB1	P27803	MNIKEFLSNKKG ASGIG
FlaB2	P27804	MKIKEFMSNKKG ASGIG
FlaB3	P27805	MLKNFMKNKKG AVGIG
M. vannielii		
FlaB1	AAC27725	MSVKNFMNNKKG DSGIG
FlaB2	AAC27726	MKITEFLNNKKG ASGIG
FlaB3	AAC27727	MMKKFLMDKKG AVGIG
M. maripaludis		
FlaB1	n/a	MKIKEFLKTKKG ASGIG
FlaB2	n/a	MKITEFMKNKKG ASGIG
FlaB3	n/a	MVKKFMKNKKG AVGIG
M. thermolithotrophicus		
FlaB1	n/a	MKIAQFIKDKKG ASGIG
FlaB2	n/a	MKIAQFIKDKKG ASGIG
FlaB3	n/a	MKIFEFLKNKKG ASGIG
FlaB4	n/a	MLKKFFKNRRG AVGIG
M. jannaschii		
FlaB1	Q58301	MKVFEFLKGKRG AMGIG
FlaB2	Q58302	MKVFEFLKGKRG AMGIG
FlaB3	Q58303	MLLDYIKSRRG AIGIG
A. fulgidus		
FlaB1-2	AAB90185	MRVGSRKLRRDEKG FTGLE
FlaB1-1	AAB90186	MGMRFLKNEKG FTGLE
H. halobium		
FlaA1	P13074	MFEFITDEDERG QVGIG
FlaA2	P13075	MFEFITDEDERG QVGIG
FlaB1	P13076	MFEFITDEDERG QVGIG
FlaB2	P13077	MFEFITDEDERG QVGIG
FlaB3	P13078	MFEFITDEDERG QVGIG
P. horikoshii		
FlaB	BAA29635	MTVVPRKG AVGIG
FlaB	BAA29637	MRRG AVGIG
FlaB	BAA29638	MKKG AVGIG
FlaB	BAA29639	MRKG AIGIG
FlaB	BAA29640	MRRG AIGIG
P. abyssi		
FlaB2	CAB50400	MKNLQGGAWQMARRG AIGIG
FlaB1-2	CAB50399	MHRKG AIGIG
FlaB1-1	CAB50398	MRRG AIGIG
A. pernix		
	BAA80910	MRRRRG IVGIE
	BAA80912	MRRRRG IVGIE

*The -3 to -1 positions, which are conserved in all archaeal preflagellins, are highlighted.

and a β turn-inducing amino acid is often found in the –4 to –6 region (Dalbey 1994). In addition, the archaeal flagellin leader peptide is much shorter than most bacterial leader peptides. However, the N-terminal leader peptides of group A type IV prepilins are short (6 or 7 amino acids) and basic. Group B type IV prepilins from the toxin co-regulated pili of *V. cholerae* and the bundle-forming pili of enterpathogenic *E. coli* are longer: 25 and 13 amino acids, respectively. The homology within this family is most apparent near the junction of the leader peptide and the mature pilin (Strom et al. 1994). As with the preflagellin examined here, prepilin cleavage occurs following a glycine residue. In the case of type IV pilins the –2 position is held by a lysine as in archaeal flagellins, while the –3 is usually glutamine (Strom and Lory 1991).

Single and multiple amino acid substitutions in the leader peptide and amino-terminal conserved region of the type IV pilin from *P. aeruginosa*, with the exception of the –1 glycine, generally had surprisingly little effect on subsequent processing by prepilin peptidase (MacDonald et al. 1993; Strom and Lory 1992; Strom and Lory 1991). Substitution of the prepilin –1 glycine with almost any other amino acid inhibited cleavage of the leader peptide and prevented assembly of the pilus (Strom and Lory 1991). Only alanine in the place of glycine allowed even partial processing of prepilin (Strom and Lory 1991). The conserved –2 lysine could be changed while still allowing proper prepilin processing. In fact, substitution of several of the residues in the short leader peptide did not affect subsequent proper processing by prepilin peptidase. Furthermore, substitutions of the +1 phenylalanine with either a polar, hydrophobic or charged residue did not affect the posttranslational processing of the prepilin and neither did the majority of other amino acid substitutions in the highly conserved N-terminal

region of the pilin. In spite of these observations, the archaeal preflagellin is not processed by the prepilin peptidase of *P. aeruginosa* unless the prepilin leader peptide is fused to the mature methanogen flagellin (Bayley and Jarrell 1999). Obviously, the preflagellin is also not processed by leader peptidases present in *E. coli*, since only unprocessed precursor is present in *E. coli* overexpressing the preflagellin.

Aside from the flagellins, to date the S layer protein is the only other *M. voltae* protein with a demonstrated leader peptide (Konisky et al. 1994). However, the 12 amino acid leader sequence (MVASALATGVFA) for the S layer protein (initially reported as an ATPase (Dharmavaram et al. 1991)) has little similarity to the archaeal preflagellin leader peptides, despite the identity in length, and specifically it lacks the conserved glycine at -1. In addition, the leader peptide is not followed by a stretch of hydrophobic amino acids but instead has acidic or basic amino acids in 8 of the first 21 positions of the mature protein. The S layer gene and protein of *M. fervidus*, another flagellated methanogen have also been studied (Bröckl et al. 1991). In this case the leader peptide has a typical bacterial like leader peptide of 22 amino acids with a sequence Ala-Gly-Ala preceding the cleavage site. This is also very unlike the leader peptides observed in the archaeal flagellins and again the conserved -1 glycine is absent. Interestingly, a glucose binding protein in *Sulfolobus solfataricus* is also produced with an 11 amino acid leader peptide that is processed at a glycyl-leucyl peptide bond. This leader peptide, as with those of the preflagellins, is also positively charged, which may suggest that this protein is secreted by a similar mechanism (Albers et al. 1999). However, the question of whether flagellins, S layer proteins and other precursors are processed by the same enzyme has yet to be experimentally determined. We would predict based on the

conservation of the amino acids around the cleavage site of the preflagellins in all archaea (Table 9) that the preflagellin peptidase is a dedicated enzyme for cleavage of preflagellin and perhaps a limited number of related proteins much as the prepilin peptidase recognizes only prepilin and pseudopilin substrates (Nunn and Lory 1992).

Over thirty N-terminal sequences of archaeal flagellins from at least fourteen different organisms are now available from either direct protein sequencing of purified protein or deduced from the gene sequence from cloned flagellins or from completely sequence genome publications (Kawarabayasi et al. 1998; Klenk et al. 1997; Jarrell et al. 1996b; Bult et al. 1996). These include flagellins from members of both archaeal kingdoms (the Crenarchaeota and the Euryarchaeota) and include mesophiles, thermophiles and hyperthermophiles. These N-termini are conserved and very hydrophobic in relation to the remainder of the molecule (Kalmokoff and Jarrell 1991; Kalmokoff et al. 1990). The sequence G(I L V)(G E D)(T A)(L A)I(V I)(F L)IA(M F L)(V I)(L V I T)(V T)A(A S)(V I)(A F L)(A S)(G A S Y)V is conserved for positions 3 to 23 of the demonstrated or presumed mature proteins. This region may be analogous to the conserved N-termini of bacterial type IV pilins. In both cases, this region may play an important role in the assembly and/or function of the pilus or flagellum, respectively. In addition, this highly conserved region may play an essential role in the recognition of preflagellin by the preflagellin peptidase. It is known in the prepilin system that the +5 glutamic acid is needed for proper methylation of the N-terminal phenylalanine residue by the prepilin peptidase (MacDonald et al. 1993; Strom and Lory 1991).

Preflagellin peptidase activity active against *M. voltae* FlaB2 was demonstrated in the membranes from every *Methanococcus* species tested, with the exception of *M. igneus*, which may not be flagellated. Comparison of the preflagellin sequences found in the heterologous methanogens to that of FlaB2 provides some preliminary data about the possible amino acid substitutions in the preflagellin which still result in proper cleavage. The lack of activity observed in membranes of *M. cariaci* and *M. marisnigri* cannot be explained as yet since the respective flagellin genes have not been sequenced. While the N-terminal sequence of the two *M. marisnigri* flagellins have been previously reported (Kalmokoff et al. 1992), the leader peptide sequences for neither *M. marisnigri* nor *M. cariaci* flagellins are known. Interestingly, preflagellin peptidase activity was detected at a comparable level in both wildtype *M. voltae* and the nonflagellated mutant *M. voltae* P-2. In the initial characterization of *M. voltae* P-2 (Jarrell et al. 1996a), western blot experiments using anti-flagellin antisera revealed the presence of a single 20 kDa crossreactive band. In comparison, in wildtype cells, 31 and 33 kDa bands (representing mature flagellins) as well as an 18 kDa band were observed. It was suggested at that time that the 18 kDa band in the wildtype membranes represented flagellin which had its leader peptide removed but which had not yet received the hypothetical posttranslational modifications that result in its final higher apparent molecular weight. In the case of the mutant, the 20 kDa band was reasoned to be flagellin that had its leader peptide still attached. As such, the preflagellin could not be modified or incorporated into flagella filaments but remained in the cytoplasmic membrane. Following this reasoning, these observations suggested that the *M. voltae* P-2 mutant would lack preflagellin peptidase

activity. Clearly, this is not the case and another explanation for the 18 and 20 kDa bands must be found.

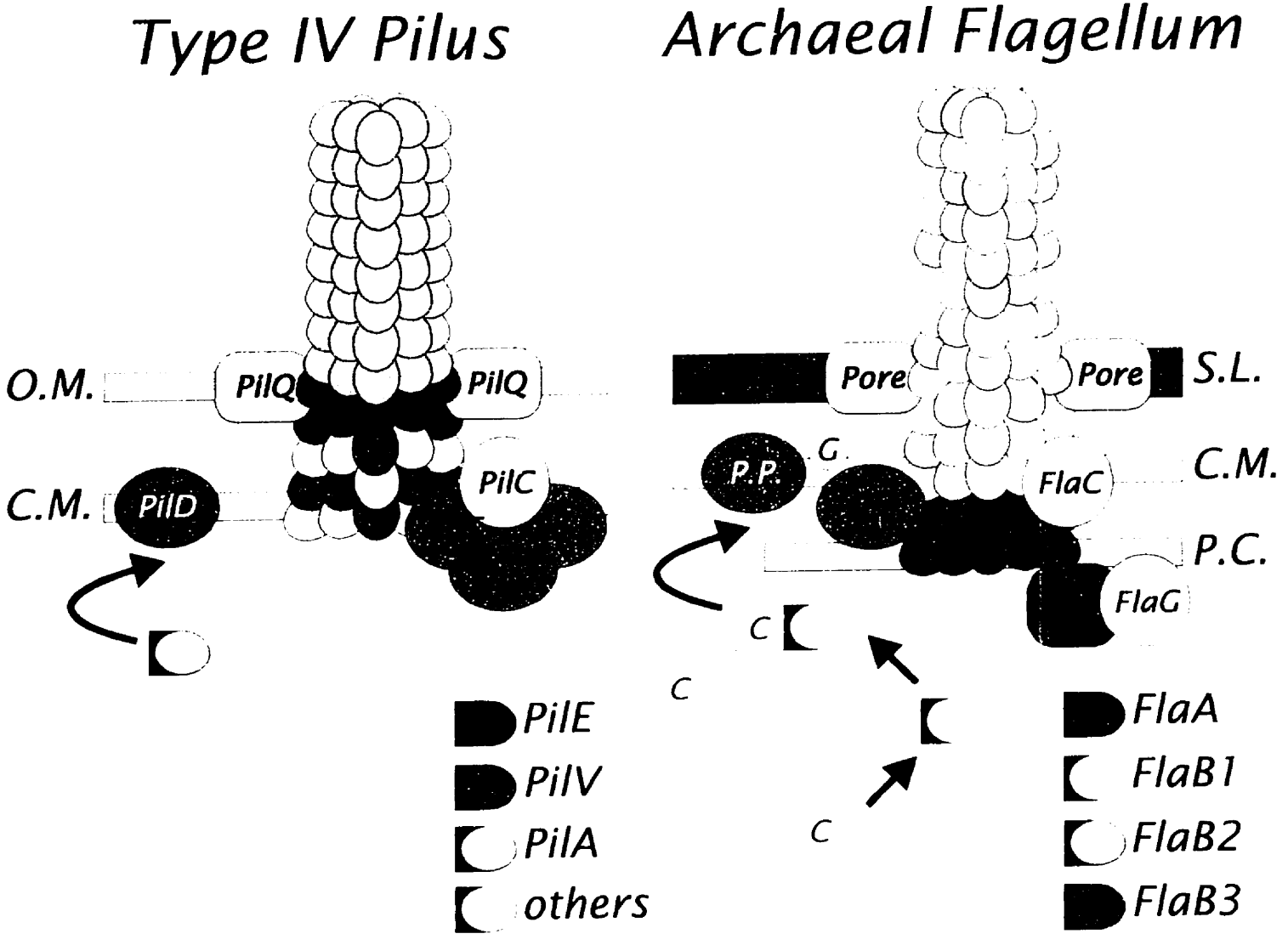
Currently, we are generating, by PCR, a family of mutant preflagellins with amino acid substitutions at the conserved positions near the cleavage site. Development of the preflagellin peptidase assay, as reported in this contribution, will allow us to determine key residues present in the preflagellin that are required for proper processing.

Unlike the assembly of bacterial flagella, it seems more likely that the flagellins of the Archaea cross the membrane via a leader peptide-dependent mechanism and then are modified (i.e. glycosylation) external to the cytoplasmic membrane (as in halobacteria) before incorporation into the filament. If so, incorporation would most likely occur at the base of the structure, like bacterial pili. Given that archaeal flagellins show similarity to type IV pilins (including the presence of a short leader peptide), demonstrate no homology to bacterial flagellins and at least one downstream gene (*M. voltae flaI*) is highly similar to the *pilT* gene of the *P. aeruginosa* type IV pilin system, a pilus-type assembly system can be envisioned for the archaeal flagellum.

A speculative model of the assembly of archaeal flagella is presented in Figure 22 with comparisons to the bacterial flagellum and type IV pilus.

Step 1. Immediately after translation, cytoplasmic chaperones may initially interact with the preflagellins to prevent the non-specific aggregation of their hydrophobic N-termini. Evidence for this step is supported by the isolation of a cytoplasmic, ATP-dependent flagellin-binding protein from the halophilic archaeon *Natronobacterium magadii* (Polosina et al. 1998).

Figure 22. Models for the assembly of the type IV pilus of *P. aeruginosa* and for the archaeal flagellum. Model for the asembly of the type IV pilus of *P. aeruginosa* adapted from Mattick and Alm (1995). In this model, the prepilin (PilA) leader peptide is removed by a membranous leader peptidase (PilD) before incorporation at the base of the of the pilus structure. Speculative model for the assembly of the archaeal flagellum is adapted from Jarrell et al. (1996b). Localization studies in *M. voltae* have identified the membranous (FlaC, FlaI) or cytoplasmic (FlaF, FlaG) destination of some flagella accessory-related proteins. The presence of a S layer pore has yet to be demonstrated in any archaeon. Details are provided within the text. C = chaperone protein, G = glycosylase, P.P. = preflagellin peptidase, S.L. = S layer, C.M. = cytoplasmic membrane, O.M. = outer membrane, P.C. = polar cap


Type IV Pilus
Archaeal Flagellum
O.M.
PilQ
PilQ
C.M.
PilD
PilC
Pore
Pore
S.L.
P.P.
G
FlaC
C.M.
P.C.
FlaG
C
C
C
PilE
PilV
PilA
others
FlaA
FlaB1
FlaB2
FlaB3

Step 2. The chaperone-preflagellin complex travels to the cytoplasmic membrane in the vicinity of the polar cap structure. At the cytoplasmic membrane, the chaperone protein is released, and the flagellin is transported through cleavage of the N-terminal leader peptide by a preflagellin peptidase. Bacterial flagellins are not made with leader peptides. However, P-ring and L-ring proteins are synthesized with N-terminal signal sequences and are exported by the same signal-peptide dependent mechanisms that are employed by other bacterial periplasmic or outer-membrane proteins (Macnab and DeRosier 1988).

Step 4. If the flagellins undergo modification such as glycosylation, this would occur external to the cytoplasmic membrane prior to their incorporation into the flagellar filament. Glycosylation may be accomplished by a dedicated glycosylase. Such a glycosylase may be one component, among others (i.e., peptidase, etc.), of an inner membrane assembly complex analogous to the machinery responsible for the processing of *N. gonorrhoeae* pilin (Fussenegger et al. 1997).

Step 5. Analogous to the pilus system, mature flagellin subunits would be incorporated at the base of the growing structure. In contrast, bacterial flagellins travel through the hollow filament and emerge for assembly at the distal end. In *M. voltae*, the filament is formed mainly by the polymerization of FlaB1 and FlaB2 preflagellins. *M. voltae* FlaI is a PilT homolog and likely provides energy for flagellar assembly or twitching motility. Termination of filament growth may occur by incorporation of minor flagellin species such as FlaA and FlaB3 in *M. voltae*. These minor flagellin subunits may form an anchoring mechanism that extends inward toward the polar membrane. This is likely equivalent to the polar cap observed in *H. salinarum* (Kupper et al. 1994).

FlaF and FlaG appear to lack the leader peptide of the flagellins but still possess a very hydrophobic N-terminus. These proteins are likely expressed at low levels due to their position in the multi-transcriptional unit. They may be analogous to PilE and PilV, which also possess very hydrophobic N-termini with sequence similarity to the major structural pilin (PilA). It seems likely that a pore (equivalent to PilQ) is required to allow the flagellum to transverse the S layer found as a wall component of many archaeal species. FlaJ of *M. voltae* may perform this function. However, various flagellated species of bacteria that also have S layers appear to lack pores at flagellar insertion points. Instead, slight irregularities in the regular pattern of the S layer are found at these sites (Sleytr and Glaubert 1975). In some cases (i.e., *Chlostridium thermohydrosulfuricum*), the diameter of the flagellar hook has about the same dimensions as the centre-to-centre spacing of the subunits in the hexagonal pattern. It appears that the flagellum replaces one or more subunits in the regular array (Sleytr and Glaubert 1975).

Future directions will involve the generation of *M. voltae* mutants for individual flagellar-related genes of the flagellin gene region using the pUC::*pac* vector system (Jarrell et al. 1996a). The development of the preflagellin peptidase assay, as described in this contribution, will be instrumental in the identification of *M. voltae* mutants lacking peptidase activity.

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