Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 154-160

CONSERVED N-TERMINAL SEQUENCES IN THE FLAGELLINS OF ARCHAEBACTERIA

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Received December 22, 1989

SUMMARY: Methanococcus voltae produces two flagellins of molecular weight 31,000 and 33,000. Amino acid analysis as well as peptide mapping with cyanogen bromide, chymotrypsin and Staphlococcus aureus V-8 protease indicates that the two flagellins are distinct. N-terminal sequencing of the 31,000 Mc. voltae flagellin as well as the 24,000 and 25,000 molecular weight flagellins of Methanospirillum hungatei GP1 shows an extensive homology with the reported N-terminus of the flagellins from Halobacterium halobium, deduced from the nucleotide sequence of the cloned genes. However, the N-termini of all three sequenced methanogen flagellins lack a terminal methionine and start at position 13 from the N-terminus of H. halobium flagellins. This initial 12 amino acid stretch may be a leader peptide which is subsequently cleaved to generate the mature flagellin, which could suggest flagellar assembly in archaebacteria occurs by a mechanism distinct from that in eubacteria. The high degree of conservation of the N-terminus of the flagellins from Mc. voltae, Msp. hungatei and H. halobium suggests an important role for this sequence, and that the archaebacteria share a common mechanism for flagellar biosynthesis.

The archaebacteria are a diverse group of microorganisms which includes the extremely anaerobic methanogens, extreme halophiles, thermoacidophiles, and other sulphur dependent organisms (1). The archaebacteria exhibit a number of unique morphological features not found among the eubacteria, including walls that lack peptidoglycan and ether-linked membrane lipids, which together result in a number of unusual cell envelope types (2). The assembly and insertion of flagella into these unusual envelopes is likely to exhibit unique features not found in the eubacterial systems. Although not a great deal is known about flagellar structure in the archaebacteria, the flagella of the extreme halophile Halobacterium halobium (3,4,5,6,7) as well as those of several methanogens (8,9) have been studied.

In general, archaebacterial flagellins are thinner (10-13 nm) than their eubacterial counterparts (20 nm,(10)). In all cases reported to date, the flagellar filaments of archaebacteria are composed of multiple

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0006-291X/90 $1.50

flagellins (two in Methanococcus voltae (9) , Methanococcus jannaschii, andMethanogenium marisnigri (M.L. Kalmokoff, S.F. Koval, and K.F. Jarrell, Abstracts Canadian Society of Microbiologists Annual Meeting, Windsor, Canada, June 19-22, 1988, Abstract MS1), 2-3 in Methanospirillum hungatei (G. Southam, M.L. Kalmokoff, K.F. Jarrell, S.F. Koval, and T.J. Beveridge, submitted for publication) and five in Halobacterium halobium (4). In addition, the flagellins of both Msp. hungatei and H. halobium are unusual in that they are glycosylated (Southam et al. submitted manuscript,6). All five flagellins of H. halobium have been cloned and sequenced and code for five closely related proteins of molecular weight 20,000 (4), all of which are found within the flagellar bundle (3).

We have determined that the two flagellins of Mc. voltae appear to be distinct; however, the N-terminus of the Mc. voltae 31,000 flagellin and the 25,000 and 24,000 flagellins from Msp. hungatei show an extensive homology with the N-terminus of the five flagellins of H. halobium. These results suggest the existence of a common mechanism for flagellar biosynthesis throughout the archaebacterial kingdom, which may be distinct from that found in the eubacteria.

MATERIALS AND METHODS

Growth of Microorganisms: Methanococcus voltae PS and Methanospirillum hungatei GP1 were obtained from G.D. Sprott (National Research Council of Canada, Ottawa, Canada). Mc. voltae was maintained at 37°C in Balch medium III (11), Msp. hungatei GP1 was maintained at 37°C in JMA medium (12). For isolation of flagellins, cultures were grown at 37°C in 1 liter bottles modified to accept serum stoppers, and pressurized daily with CO_2/H_2 (1:4, v/v).

Isolation of flagella: Cells were harvested in early stationary phase by centrifugation (6000 X g, 4°C) and gently resuspended into 150 ml of 100 mM Tris-HCl (pH=7.5), containing 150 mM NaCl and sheared in a Waring Blender for 90 seconds. Following a low speed centrifugation (6000 X g for 15 minutes) to remove whole cells, and further clarification by centrifugation at 16,000 X g / 1 hour, the flagellar filaments were recovered by high speed centrifugation at 80,000 X g / 90 minutes. Pellets were stored frozen at -20°C.

Electophoresis: SDS-PAGE was performed according to the method of Laemmli (13).

Peptide Mapping: One dimensional peptide mapping was performed according to the method of Cleveland et al. (14). Cyanogen bromide cleavage was carried out according to Lam and Kasper (15).

Sequence Analysis: Samples for amino acid analysis and N-terminal sequencing were prepared by the method of Hunkapiller et al. (16). Analysis was carried out using an Applied Biosystems 470A Gas Phase Protein Sequenator with an in-line PTH amino acid analyzer (Applied Biosystems 120A) by M. Blum (Dept. Biochemistry, University of Toronto, Toronto, Canada). Sequences were compared for conservative substitutions using the Lipman and Pearson algorithm (17).

RESULTS AND DISCUSSION

Peptide mapping of the 33,000 and 31,000 flagellins from Mc. voltae with cyanogen bromide, chymotrypsin and Staphlococcus aureus V-8 protease

indicated the distinct nature of the two proteins (Figure 1). Cyanogen bromide cleavage completely degraded the 33,000 flagellin and produced three peptides detectable within this gel system from the 31,000 flagellin, indicating a significant difference in terms of methionine content between the two flagellins. No apparent homology was detected in partials generated using either chymotrypsin or S. aureus V-8 protease. Amino acid analysis of the two flagellins (Table 1) also supports this finding. Threonine, serine and valine content of the 33,000 flagellin are significantly higher than of the 31,000 flagellin, and the aspartic acid content of the 31,000 flagellin excedes that of the 33,000 flagellin. Taken together, the data indicates that the 33,000 flagellin is not a read through product of the 31,000 flagellin. H. halobium codes for five different flagellins; however, they are very closely related, coding polypeptides of molecular weight of 20,000 (4).

N-terminal sequencing of the two Mc. voltae flagellins was attempted. The N-terminal 19 residues of the 31,000 flagellin were obtained but the N-terminus of the 33,000 flagellin was blocked. The N-terminal sequence of the 31,000 flagellin of Mc. voltae showed no homology with any of the

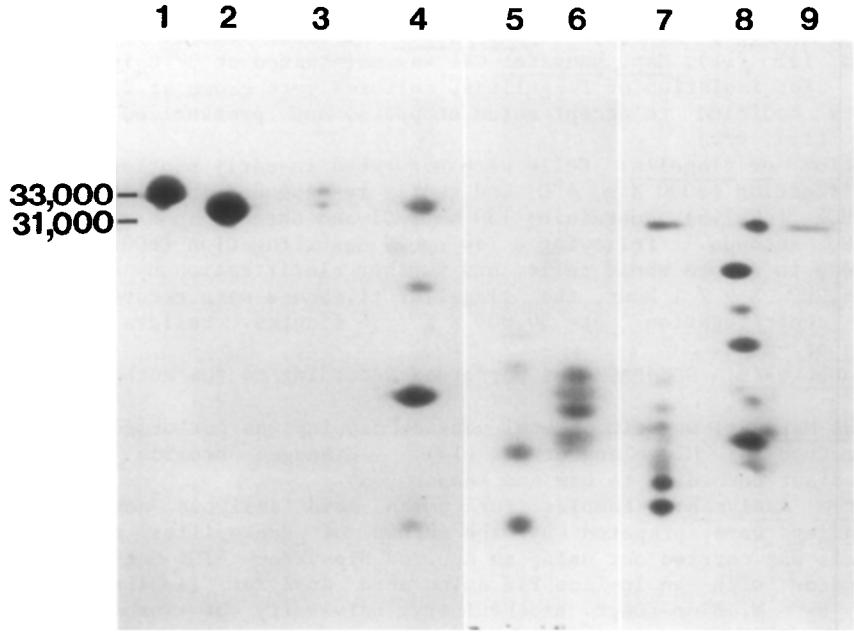

FIGURE 1. Peptide mapping of the 33,000 and 31,000 flagellins of Methanococcus voltae. Lane 1: 33,000 flagellin Lane. 2: 31,000 flagellin. Lane 3,4: Each respective flagellin treated with cyanogen bromide. Lane 5,6: Each respective flagellin treated with chymotrypsin (5ug). Lane 7,8: Each respective flagellin treated with Staphlococcus aureus V-8 protease (2.4 ug). Lane 9: S. aureus V-8 protease alone. Samples were separated on 15% SDS-PAGE gels.

TABLE 1

Amino acid composition of the 33,000 and 31,000 flagellins of Methanococcus voltae

Amino acid	Residues per molecule	
	33,000	31,000
Asp	34	46
Thr	26	11
Ser	32	12
Glu	39	36
Pro	15	13
Gly	40	37
Ala	37	41
Cys	0	0
Val	24	7
Met	ND*	ND
Ile	17	23
Leu	23	21
Tyr	2	2
Phe	9	11
His	5	3
Lys	19	23
Arg	5	4

* ND, not determined.

reported eubacterial flagellins, yet extensive homology was found with the published N-terminus of the flagellins from H. halobium deduced from the sequence of the cloned genes (Figure 2). An exact match was found in 15 of 19 residues, and allowing for conservative substitutions, homology within this region is 90%. This finding is remarkable considering that these two organisms are distantly related and encompass two major branches of the archaebacterial kingdom. The N-terminal sequence of two of the flagellins from Msp. hungatei GP1 (25,000 and 24,000) were also obtained. Results comparing all three N-terminal sequences are shown in Figure 2. The N-terminal sequence of the 25,000 flagellin of Msp. hungatei GP1 was identical to the 24,000 flagellin over the first 20 residues that were determined. Of the 32 residues sequenced in the 24,000 flagellin, 13 were identical to the H. halobium sequence, and when conservative substitutions were taken into account, homology was 84%. All three archaebacterial sequences are very hydrophobic.

None of the three sequenced methanogen flagellins had an N-terminal methionine, and all three start at the same internal position on the deduced H. halobium flagellin sequence (residue 13). This finding suggests that the flagellins may have undergone a post-translational modification. The first 12 amino acids may represent a leader sequence. Unfortunately, direct comparisons with the N-terminal sequences for the

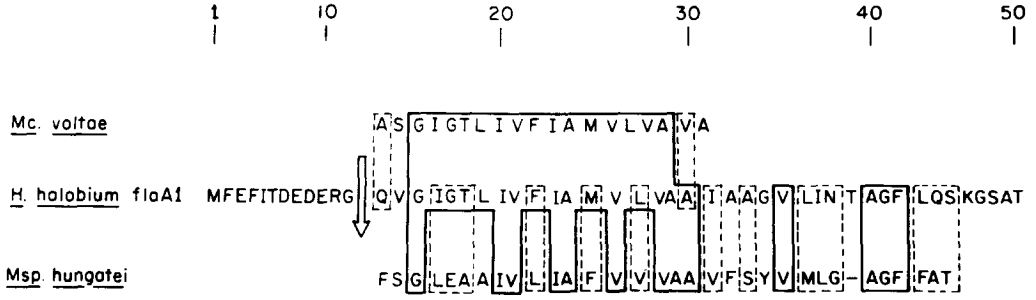

FIGURE 2. Comparison of the N-terminal sequences from the 31,000 flagellin of Mc. voltae, H. halobium FlaA1 (4), and the 24,000 flagellin of Msp. hungatei. Solid line indicates exact matches of amino acids, dashed lines are conservative substitutions. Arrow marks the cleavage site of the putative leader peptide.

flagellins of H. halobium cannot be made as the mature Fla I flagellin was resistant to Edman degradation (4). The putative leader sequence of H. halobium bears no obvious homology to the published 34 amino acid leader sequence of the halobacterial cell surface glycoprotein (18) or the leader peptide of bacteriorhodopsin, which is 13 amino acids long (19).

This extensive homology found in the N-terminus of the flagellins from these three archaebacteria is interesting, particularly in light of the fact that the flagellins show a marked variation in terms of molecular weight and the environment in which they must function (ranging from low to medium to extremely high levels of NaCl). In addition, the flagellins of Mc. voltae appear to be quite different from each other, while the flagellins of H. halobium are closely related, glycosylated and significantly smaller than those of Mc. voltae. Conservation of the N-terminal region suggests that this sequence is of some importance, perhaps indicating a common mechanism of flagellar biosynthesis among the archaebacteria. At this point we are not certain how extensive the homology may be between the rest of the flagellin molecule or if it is limited to the N-terminal region. In eubacteria, there is homology among diverse species in the N and C terminal regions of the flagellins (20), regions of the proteins believed to be important in polymerization and migration of the flagellin up the filament (21).

In eubacteria, flagellin transport across the cytoplasmic membrane is thought to occur by a flagella specific pathway (22). Flagellins do not have leader sequences, but appear to pass up through the core of the flagellar filament for addition to the distal end of the growing filament. Proteins associated with the basal body (i.e. P and L rings) do have signal sequences and are transported across the membrane in the usual fashion (23). Putative signal sequences have been reported on the periplasmic

flagellins of Spirochaeta aurantia (24) and possibly in Treponema (25). These sequences bear no homology to the archaebacterial flagellin N-terminal sequences. The assembly of these unusual flagella has not been described.

Our findings indicate that the N-terminal region of the flagellins of three members of two branches of the archaebacterial kingdom are highly conserved. In addition, these flagellins may initially be synthesised with a leader sequence since the methanogen flagellins all lack a terminal methionine and start at the same internal position of the H. halobium flagellin sequence. The very conserved and hydrophobic N-termini of the mature flagellins of both methanogens and H. halobium suggests that they may be regions important in flagellin filament assembly. Sumper and coworkers (4,5) have reported that glycosylation of the H. halobium flagellins occurs at the extracellular surface of the plasma membrane, which suggests that polymerization into a functional flagellum may occur by a mechanism quite different from that found in the eubacteria (4). The presence of a leader sequence would support this hypothesis and raises very interesting questions concerning flagellar assembly in this kingdom. The archaebacterial flagellin may be translocated across the cytoplasmic membrane (rather than through the hollow centre of the filament) using the leader peptide, subsequently modified if required (i.e. glycosylation as found in both H. halobium and Msp. hungatei) and finally inserted into the growing filament.

We are currently cloning and sequencing the Mc. voltae flagellins to conclusively demonstrate the presence of a leader peptide and addressing the question of polarity of growth of the archaebacterial flagellar filament.

ACKNOWLEDGMENTS: This work was supported by a Natural Sciences and Engineering Research Council of Canada Operating Grant to K.F.J. M.L.K. was a recipient of an Ontario Graduate Scholarship. The authors wish to thank John Kuzio for sequence analysis of flagellin N-terminal regions.

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