Giant flagellins form thick flagellar filaments in two species of marine Gammaproteobacteria

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Abstract

Flagella, the primary means of motility in bacteria, contain thousands of copies of the protein flagellin in self-assembled helical filaments that function as microscopic propellers. Evolution has presented a wide range of different sizes of flagellin, but the upper reaches of the size distribution have barely been explored. We show here that two species of marine Gammaproteobacteria, *Bermanella marisrubri* Red65 and *Oleivorans marinus* 2O1, are motile due to the production of thick, monopolar flagellar filaments. In each case, a 'giant' flagellin of more than 1,000 amino acids is the only predicted flagellin protein. Two species of *Methylobacterium* from the leaves of *Arabidopsis thaliana* also both possess genes for giant flagellins. However, their flagellar filaments are of similar thickness to bacteria with flagellins half the size. This may be explained by the presence of multiple, smaller, flagellin genes in the *Methylobacterium* species. This work further illustrates how "the" bacterial flagellum is not a single, ideal structure, but a continuum of evolved machines adapted to a wide range of niches.

Introduction

Flagella are the organelles responsible for motility in a large and diverse array of bacterial species. They take the form of helical filaments, several microns long, connected to a basal body that spans the cell envelope (1). Some species have only a single flagellum, while others possess multiple filaments that form a coherent bundle for swimming (2). Flagellar motors consist of over 20 different proteins that harness proton- or sodium-motive forces to generate torque (3). The motor spins the flagellar filament to propel the cell.

The flagellar filament is composed of thousands of flagellin monomers. Flagellins are secreted by the flagellar type III secretion system and travel through the hollow core of the growing filament to assemble into a helical array at the distal tip (4). Flagellins from diverse species all possess conserved N- and C-terminal regions that fold together to form D0 and D1 domains (2, 5). These domains are the basis of extensive inter-flagellin contacts, polymerizing to form the inner tubular section of the flagellar filament. The central domain of the protein forms the surface-exposed region of the filament and is far more variable, both in sequence and in length (6).

Given the conserved nature of the D0 and D1 domains, it has generally been assumed that flagellar filaments are all packaged in similar ways. Work has particularly focussed on *Salmonella enterica* ssp. *enterica* sv. Typhimurium (*S.* Typhimurium) and *Escherichia coli*. However, recent discoveries have revealed much greater variation in flagellar structure than was previously appreciated (7–9).

Most work on flagellins to date has focussed on proteins of a similar size to that of the phase 1 flagellin (FliC) of *S.* Typhimurium (495 aa; 51.6 kDa). However, this leaves open the question of how large a flagellin can be and still assemble into a flagellar filament. We suspected that the answer to this question might best be found by surveying the sizes of flagellins occurring in nature. We therefore performed a database search to identify 'giant' flagellins greater than 1,000 aa in length. We hypothesised that if giant flagellins are expressed and assemble into flagellar filaments, then the resulting filaments might be larger than the canonical flagellar filaments from *S.* Typhimurium. We therefore undertook an experimental investigation of filament thickness in strains that potentially encode giant flagellins. We selected two sets of aerobic, mesophilic bacterial strains from the German Culture Collection (DSMZ): two species of *Methylobacterium* isolated from the leaves of *Arabidopsis thaliana* (10) and two hydrocarbon-degrading marine Gammaproteobacteria that colonise oil spills: *Bermanella marisrubri* Red65, originally isolated from the Red Sea (11) and *Oleibacter marinus* 2O1, originally isolated from Indonesian seawater (12). We used transmission electron microscope (TEM) and light microscope observations to investigate the thickness of flagellar filaments expressed during growth in liquid medium.

Materials and Methods

Database searches

Using the UniProtKB database, we accessed all known protein sequences that harbour the PFAM domain pf00669, which represents the N-terminal helical domain common to

all flagellins and to some representatives of the hook-filament junction protein FlgL. We ranked the proteins by size (http://www.uniprot.org/uniprot/?query=pf00669&sort=length).

Strains and Growth Conditions

The strains of bacteria used in this study are detailed in Table 1. All four strains were obtained as live cultures from the DSMZ.

All cultures were grown in 5 mL of medium within loosely-capped 30 mL sterile polystyrene universal tubes at 28 °C and shaking at 200 rpm. *Methylobacterium* spp. Leaf90 and Leaf123 were both grown in DSMZ medium 1629, which contains (per L) 1.62 g NH_4Cl, 0.20 g $MgSO_4.7H_2O$, 2.40 g K_2HPO_4, 0.96 g $NaH_2PO_4.H_2O$, 15 mg Na_2EDTA, 3 mg $FeSO_4.6H_2O$, 4.50 mg $ZnSO_4.7H_2O$, 3 mg $CoCl_2.6H_2O$, 0.64 mg $MnCl_2.4H_2O$, 1 mg H_3BO_3, 0.40 mg $Na_2MoO_4.2H_2O$, 0.30 mg $CuSO_4.2H_2O$, 3 mg $CaCl_2.6H_2O$ and 5 mg methanol. All components of this medium were purchased from Sigma-Aldrich (Missouri, U.S.A.). The culture medium for *B. marisrubri* and *O. marinus* was Marine Broth 2216 (BD, New Jersey, U.S.A.) supplemented after autoclaving with 1% filter-sterilised Tween 80.

Table 1: Bacterial strains used in this study

Strain	**DSMZ Accession No.**	**Giant flagellin Uniprot entry**	**Large flagellin length (aa)**	**Proteins with pf00669 in genome**		**Ref**
				Total	**Annotated as flagellin**	
Methylobacterium sp. Leaf123	DSM 102638	ADADQ5B6S6	1,232	7	6	(10)
Methylobacterium sp. Leaf90	DSM 102626	ADADQ4WLB4	1,449	4	3	(10)
Bermanella marisrubri Red65	DSM 23232	Q1N2Y3	1,020	2	1	(11, 13, 14)
Oleibacter marinus 2O1	DSM 24913	ADA1N7LLL1	1,190	2	1	(12)

Light and Transmission Electron Microscopy

Pre-cultures of each strain were grown as described above for 72 h. A 50 µL portion of each culture was then transferred to 5 mL of the same culture medium and grown as described above, for 24 h. Motility was screened by placing a 5 µL drop of each culture on a microscope slide and examining with differential interference contrast through a 100× oil-immersion objective lens.

For TEM, 1 mL aliquots of the fresh cultures were washed twice by pelleting the cells at 1,500 × g for 3 min and gently re-suspending in 2-(N-morpholino)ethanesulfonic acid (MES) buffer. The final resuspension used 200 µL of MES buffer to achieve a higher cell density. 4 µL of the final cell suspension was deposited on glow discharged, carbon-coated TEM grids and the cells were allowed to settle onto the surface for 1 min. Cells were negatively stained by uranyl acetate and then imaged with a Tecnai T12 transmission electron microscope with TVIPS camera.

Results and Discussion

We were surprised to discover several hundred putative large flagellins with more than 800 aminoacids and over eighty putative 'giant' flagellins greater than a thousand amino acids in length. The giant flagellins were scattered across a range of bacterial phyla and sub-phyla, including the Aquificae, Chrysiogenetes, Deferribacteres, the alpha, gamma and delta/epsilon subdivisions of the Proteobacteria, Planctomycetes, Synergistetes and the Firmicutes, plus the candidate phylum Glassbacteria from the recently described candidate phyla radiation (15).

In almost all cases, we found that the putative giant flagellins had the domain structure expected of a flagellin, with the conserved N- and C-terminal helical domains flanking a flagellin hypervariable region. However, we cannot exclude the possibility that some of these proteins might be more closely related to FlgL than to flagellin *per se.*

In our experimental investigations, all four species proved to be motile under the growth conditions that we used (Supplementary files 1 – 4). For the two *Methylobacterium* species and *O. marinus*, only a minority of the cells visible under the light microscope displayed swimming motility. However, almost all *B. marisrubri* cells were highly motile and swam extremely quickly. The *Methylobacterium* strains displayed an unusual pattern of movement, characterised by abrupt reversals of the direction of travel with no tumbles or re-orientation of the cell body.

Under the electron microscope the proportions of flagellated cells were similar to the motile proportions under the light microscope, suggesting that differences in motility were due to differential expression of the flagellar apparatus. Both *Methylobacterium* species were short rods with a single flagellum (Figure 1). Figure 1 also shows that *B. marisrubri* cells were very thin, approximately 2 – 3 µm in length, with a spiral shape and a single, polar flagellum, while *O. marinus* were shorter (1 – 2 µm), curved or spiral in shape, also with a single, polar flagellum.

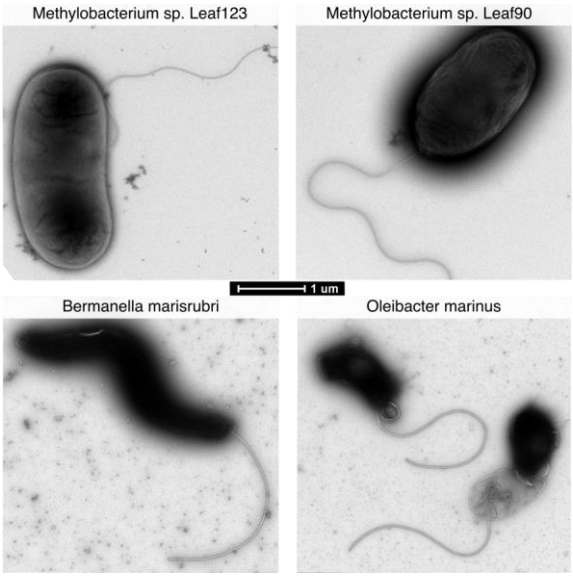

Figure 1: Negatively stained TEM images of each of the bacterial species used in this study. The images are typical of the cell morphology and flagellation pattern that we observed for each strain.

In support of our hypothesis, we found that both *B. marisrubri* and *O. marinus* produced flagellar filaments with widths of at least 30 nm (Figure 2). This is approximately 25% thicker than the Phase 1 flagellin of *S.* Typhimurium (24 nm), which is composed of 495 amino acids (5). The *B. marisrubri* and *O. marinus* genomes each encode 2 proteins annotated to contain the pf00669 PFAM domain. In each case, the putative giant flagellin is the only protein predicted to belong to the flagellin family. The smaller (412 aa in each case) proteins show greater similarity to flagellar hook-associated protein FlgL than to flagellin. Therefore, based on the predicted protein sequences and their homology to known proteins, it is likely that the putative giant flagellins are the structural components of the thick flagellar filaments seen in this study.

Contrary to expectations, *Methylobacterium* sp. Leaf123 and sp. Leaf90 had filaments of approximately the same thickness as *S.* Typhimurium (Figure 2). However, their respective genomes encode 7 and 4 proteins annotated with the flagellin N-terminal domain. Significantly, the putative large flagellins in these species show homology only to the N-terminal domain of flagellin, but not to the similarly conserved C-terminal domain. The other putative flagellins range in size from 350 – 595 aa and all contain both the N- and C-terminal domains. Therefore, it is possible that the flagellin expressed by *Methylobacterium* — at least under these growth conditions — does not fall into our category of giant flagellins.

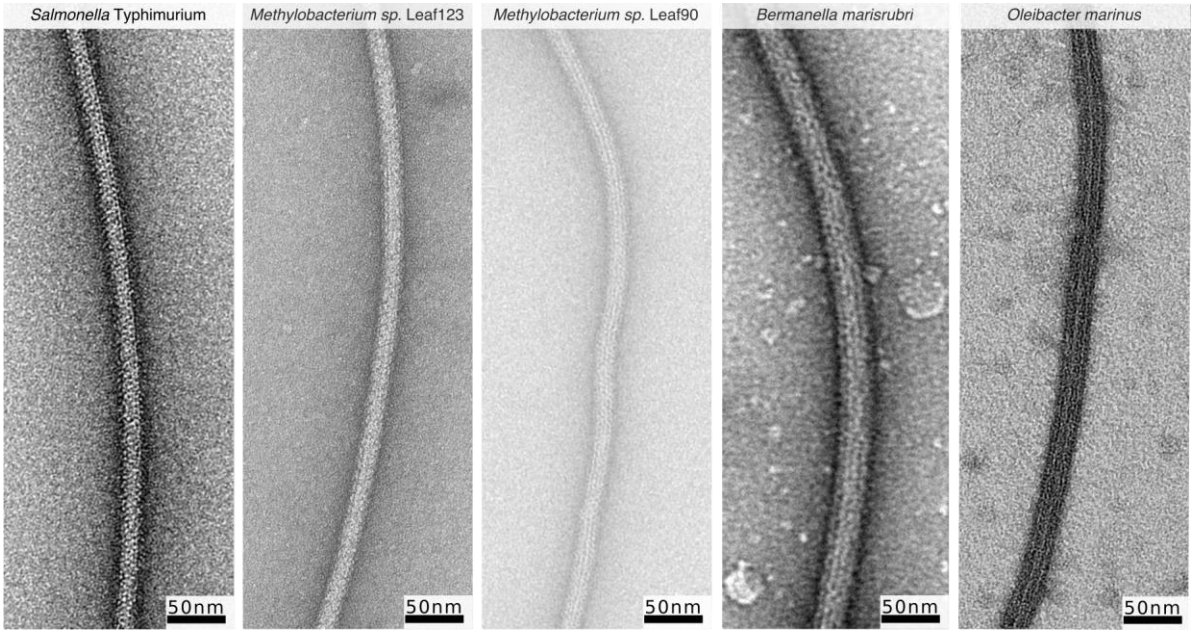

Figure 2: Negatively stained TEM images of representative flagella filaments for each of the bacterial strains studied. A filament (24 nm diameter) from S. Typhimurium *is also shown for comparison. All images are to the same scale.*

The functional advantages of giant flagellins and/or thick flagella are presently unclear. Other species of bacteria, including *Vibrio parahaemoliticus* and *Bradyrhizobium japonicum* express single, thick, polar or subpolar flagella for swimming motility in liquid media and multiple, thinner, lateral flagella for swarming on surfaces or in viscous media (16–19). However, *B. marisrubri* and *O. marinus* are not predicted to

express any other flagellins and so probably rely solely on thick flagella for their motility.

Thick flagella could represent a mechanical adaptation to allow faster or more efficient swimming. An alternative explanation is the expanded central domain of the protein confers a second function beyond motility, particularly as flagellins with active metallopeptidase enzyme domains have recently been discovered (20). However, the giant flagellins reported here do not possess sequence similarity to any enzyme domains.

The discovery of thick, polar flagellar filaments in two species of marine bacteria expands the known diversity of flagellar architecture but also raises more questions. Are other components of the flagellar apparatus correspondingly large? Do thick flagella require alternative structural arrangements to those seen in the common model organisms (3, 7)? How common or widespread is this pattern of flagellation and how did it evolve? In conclusion, this work illustrates how "the" bacterial flagellum is not a single, ideal structure, but is instead a continuum of evolved machines adapted to a wide range of niches (21–23). It will be interesting to learn how the structure and function of these marine flagellar systems are related to their sequences, and how the structures are influenced by their environments.

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