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A Deletion Variant Study of the Functional Role of the *Salmonella* Flagellin Hypervariable Domain Region in Motility

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The eubacterial flagellum is a complex structure with an elongated extracellular filament that is composed primarily of many subunits of a flagellin protein. The highly conserved N and C termini of flagellin are important in its export and self-assembly, whereas the middle sequence region varies greatly in size and composition in different species and is known to be deletion-tolerant. In *Salmonella typhimurium* phase 1 flagellin, this "hypervariable" region encodes two solvent-exposed domains, D2 and D3, that form a knob-like feature on flagella fibers. The functional role of this structural feature in motility remains unclear. We investigated the structural and physiological role of the hypervariable region in flagella assembly, stability and cellular motility. A library of random internal deletion variants of *S. typhimurium* flagellin was constructed and screened for functional variants using a swarming agar motility assay. The relative cellular motility and propulsive force of ten representative variants were determined in semi-solid and liquid medium using colony swarming motility assays, video microscopy and optical trapping of single cells. All ten variants exhibited diminished motility, with varying extents of motility observed for internal deletions less than 75 residues and nearly complete loss of motility for deletions greater than 100 residues. The mechanical stability of the variant flagella fibers also decreased with increasing size of deletion. Comparison of the variant sequences with the wild-type sequence and structure indicated that all deletions involved loss of hydrophobic core residues, and removal of both partial and complete segments of secondary structure in the D2 and D3 domains. Homology modeling predicted disruptions of secondary structures in each variant. The hypervariable region D2 and D3 domains appear to stabilize the folded conformation of the flagellin protein and contribute to the mechanical stability and propulsive force of the flagella fibers.

Keywords: *Salmonella typhimurium*; flagellin; motility; hypervariable region; optical trapping

Introduction

The eubacterial flagellum is the primary organelle for cellular propulsion in liquid environments.[1–5] Other biological functions of flagella include host adhesion, colonization and virulence.[5,6] The flagellum is a complex structure composed of more than 20 component proteins[2,7–10] and has several major features: a basal body, a transmembrane motor, a hook structure, and an elongated helical filament. The filament has an outer diameter of 12–25 nm, a 2–3 nm diameter inner channel,[10,11] and can be 1–15 μm or more in length.[9,12] It is composed of up to 30,000 subunits of a globular protein termed flagellin.[13,14]

Salmonella enterica serovar Typhimurium (*Salmonella typhimurium*) is a peritrichously flagellated

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Abbreviations used: Amp, ampicillin; LB, Luria-Bertani broth; TEM, transmission electron microscopy.

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bacterial pathogen that serves as a model organism for the investigation of bacterial flagella structure and function.[10,11,15] The mature *S. typhimurium* phase 1 flagellin protein, encoded by the *fliC* gene, consists of 494 amino acid residues; the first methionine residue is removed post-translationally. The flagellin structure (PDB 1UCU)[12,16] shows four distinct globular domains, termed D0, D1, D2 (with subdomains D2a and D2b) and D3. Domain D0 forms the inner core of the filament, D1 forms the outer core, and domains D2 and D3 form a knob-like projection on the filament surface. The polypeptide chain starts at domain D0, proceeds through domains D1 and D2, forms the outermost D3 domain, and then returns through domains D2 and D1, ending in domain D0.[16] The N and C-terminal D0 and D1 domain regions are highly conserved across different bacterial species,[17–20] as reviewed by Beatson *et al*.[21] These terminal domain regions are essential for self-assembly and are composed largely of α-helical coiled-coils that associate to form the interior of the flagella filament. The flagellin middle sequence region encodes the outer D2 and D3 domains, which are composed largely of β-sheet structures. This "hypervariable" sequence region varies greatly in size and composition in different strains and species, resulting in a wide range of observed flagellin molecular masses that vary from 20–77 kDa.[22–26] The D2 and D3 domains have been attributed with functions such as aiding in increase of traction while swimming and evasion of host defenses due to their sequence variability.[27–29] These outer domains are an important immunological determinant of this protein; the sequence of the middle region has long been correlated with variations in the H antigenicity.[23,30–34] Large segments of the D2 and D3 domains can be deleted without disabling the export and self-assembly functions of flagellin completely.[19,34–36] Furthermore, foreign peptides and proteins can be inserted into this region. This deletion and insertion tolerance has been exploited for the extracellular display of peptides on bacterial flagella.[37–42]

The role of the D2 and D3 outer domain region of flagellin in propulsion remains unclear. Further understanding of this region's deletion tolerance and function in folding, stability and self-assembly to form flagella fibers and the resulting impact on cellular motility are of fundamental interest. Structural information about functional deletion variants may be useful in developing rational design approaches for the insertion and display of fusion peptides and proteins on flagella fibers, which have potential applications as a bionanotube.[43] Here, we present the results of experimental and theoretical structure-function studies of the effects of internal deletions in the hypervariable region of *S. typhimurium* flagellin.

Results

Identification of functional *fliC* internal deletion variants

A plasmid library of random internal deletion variants of the *S. typhimurium fliC* gene was screened for functional variants *via* a swarming motility assay (Materials and Methods) with the otherwise non-motile SJW134 *S. typhimurium* strain (Table 1). Approximately 12,000 single colonies were screened, yielding about 200 functional deletion variants. A subset of 46 variants with more substantial deletions were identified by colony PCR; DNA sequencing of these variants yielded 39 unique internal deletion variants (Table 2).

Table 1. Strains and plasmids used in this study

	Description	Source/reference
A. *Strains*		
E. coli		
XL1-Blue	Cloning strain *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* {F' *proAB lacI*qZΔ*M15* Tn*10* (Tetr)}.	Stratagene
S. typhimurium		
SJW1103	LT2 derivative that is wild-type for motility and chemotaxis, phase 1 flagellin stable (*fliC* gene)	62–64
SJW134	Non-motile, flagellin-deficient strain, Δ*fliC* and Δ*fljB*, derived from parent strain SJW806	63,65,66
JR501	Stable restriction-deficient (r^-), methylation-proficient (m^+) galE cloning strain for providing restriction compatibility with *E. coli* plasmids	67,68
B. *Plasmids*		
pTH890	pTrc99A-derived Amp^r expression plasmid for wild-type *S. typhimurium fliC* gene	71,72
pTH890-C3	Derivative of pTH890, expressing FliC Δ282-293	This study
pTH890-C4	Derivative of pTH890, expressing FliC Δ261-293	This study
pTH890-C9	Derivative of pTH890, expressing FliC Δ245-291	This study
pTH890-C11	Derivative of pTH890, expressing FliC Δ231-299	This study
pTH890-C12	Derivative of pTH890, expressing FliC Δ220-328	This study
pTH890-C37	Derivative of pTH890, expressing FliC Δ250-304	This study
pTH890-C79	Derivative of pTH890, expressing FliC Δ250-324	This study
pTH890-C89	Derivative of pTH890, expressing FliC Δ250-298	This study
pTH890-C131	Derivative of pTH890, expressing FliC Δ201-368	This study
pTH890-C150	Derivative of pTH890, expressing FliC Δ227-403	This study