Rescue of bacterial motility using two and three-species FliC chimeras

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Abstract

The bacterial flagellar filament acts as a propeller to drive most bacterial swimming. The filament is made of flagellin, known as FliC in *Escherichia coli*. FliC consists of four domains, the highly conserved core D0 and D1 domains and the hypervariable outer D2 and D3 domains. The size and structure of the outer domains varies, being completely absent in some bacterial species. Here, we sought to identify outer domains from various species that are compatible with the ability of *E. coli* K-12 FliC to form filaments capable of supporting motility. We calculated a phylogeny of 210 representative flagellin amino acid sequences and generated a series of FliC variants, including outer-domain-deleted forms and eleven chimeric FliC mutants using domains from *E. coli K-12*, *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *Collimonas fungivorans*, *Helicobacter mustelae* and *Mesorhizobium* sp. ORS3359 in various combinations. Notably, two of the chimeric *fliC* mutants rescued motility in a *fliC*-disrupted *E. coli* K-12 strain, both of which contained the *S.* Typhimurium D2 domain. Overall, we demonstrate that, while most FliC chimeras did not support motility, interchangeability of the outer domains can produce filaments that provide motility, providing insights to guide the design of synthetic flagellins.

Importance

Flagellin is a key protein forming the filament of the bacterial flagellar motor which powers most bacterial swimming. Flagellin can have hypervariable domains which can alter motility in different environments and provide immune evasion. Here we engineered two flagellin chimeras that could drive motility. This indicates that the flagellin outer domains can be exchanged, to some degree, allowing us to refine rational design approaches for engineering of bacterial swimming. Our work shows the challenges to overcome when combining flagellins from different species and provides evidence that domain switched flagellins can form filaments.

Introduction

Motility provides organisms the ability to exploit new niches, to evade predation and to find increased opportunities for the exchange of genetic material (1). The flagellar filament is a major component of the bacterial flagellar motor (BFM) taking the form of the bacterial flagellum that consists of a long helical protein filament which is formed from approximately 30,000 flagellin subunits, known as FliC in *Escherichia coli* K-12 (2,3). FliC interacts with both the flagellar hook junction proteins (FlgK and FlgL) and filament cap protein (FliD) (4–6).

FliC from *E. coli* K-12 is comprised of four domains, D0, D1, D2 and D3 (7,8). Domains D0 and D1 are found on the N- and C-termini of the protein and are highly conserved across multiple bacterial phyla, such as *Proteobacteria*, *Bacillota* and *Actinobacteria* (9). These “core” domains are formed of α-helices

and interact with each other as part of the supercoiled filament (7,8,10,11). Within the D0 domain is a 22-residue segment which is the recognition signal for the flagellar export chaperone, FliS (12). Domains D2 and D3 in *E. coli* K-12, also termed "outer domains", are variable in both sequence and length even at a species level (3). These outer domains consist primarily of β-sheets and were shown in *Salmonella* Typhimurium to have no interactions between each other (7,13). In some cases, flagellin monomers from other bacterial species such as *Bacillus cereus*, *Agrobacterium tumefaciens* and *Treponema pallidum* do not contain a D2 or D3 domain but still produce a functional flagellar filament capable of motility (9,14–18). It has been shown that within the genera *Escherichia* and *Salmonella* there is a large level of diversity in the outer domains, suggesting that the outer domains are acquired via the process of evolution (19,20). This implies that the outer domains of flagellin from diverse species can be incorporated into host flagellins to provide competitive advantages that increase efficiency of motility in different environments or that allow evasion of recognition by the immune system.

In *Pseudomonas aeruginosa* PAO1 the outer domains of flagellin increase the motility of bacteria in more viscous environments due to inter-domain interactions (21). Likewise, the outer domains *E. coli* O157:H7, *E. coli* O127:H6, *Achromobacter* (which have an additional "D4" domain) and *Sinorhizobium meliloti* (which has only a D2 outer domain) can dimerise or tetramerise to form a screw-like surface which increases motility through stabilisation an intermediate waveform and prolonging tumbling (22). Furthermore, interactions of the outer domains of *E. coli* can increase tumbling time (22). Inter-species flagellin chimeras between *Salmonella* strains (serovar Typhimurium strain 14028s and serovar Enteritidis strain P125109) were able to form functional filaments (23). Of 13 FliC variants, four were motile, and it was shown that alternating the outer domains could impact motility (23).

One of the main research focuses on the flagellin outer domains is their use in immune activation and protein display. *S.* Typhimurium FliC is capable of activating the Toll-Like Receptor 5 (TLR5) (24). In a comprehensive review by Hajam *et al*., the authors identified 29 studies where flagellin was used as an immune adjuvant for bacterial, viral, parasitic and other miscellaneous antigens (25). This use of recombinant flagellin as a surface-display tool for immune activation has led to a number of vaccines being developed and used in human clinical trials (26,27). However, although chimeric FliC has been used in immunological studies, the effect of manipulating the outer domains on bacterial motility is not well understood.

Here, we tested the ability of chimeric FliC to support motility where we replace the outer domains of *E. coli* K-12 with outer domains from flagellins of a diverse set of bacterial. We show that the outer domains of *E. coli* K-12 FliC are not required to drive swimming motility and that two forms chimeric FliC can form functional filaments that support swimming motility.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Table S1 describes the strains and plasmids used in this study. The *E. coli* strain SYC29 was constructed from the chemotactic wild-type strains RP437, using the λ RED recombination system, as previously described (28,29). *E. coli* strains were grown in Lysogeny Broth (LB) (10 g/L NaCl, 10 g/L Bacto-tryptone, 5 g/L Yeast Extract). For 2% (w/v),0.3%, 0.35% and0.4% (w/v) LB agar plates 20 g/L, 3 g/L, 3.5 g/L or 4 g/L agar was added, respectively. *E. coli* strains were grown in Tryptone Broth (TB) to aid in induction of motility (5 g/L NaCl, 10 g/L Bacto-tryptone). Antibiotic selection used ampicillin (AMP) (50 µg/ml), chloramphenicol (CAM) (25 µg/ml) and/or tetracycline (TET) (10 µg/ml). Induction for gene

85 expression from plasmids required L-arabinose (0.02% (w/v)) and/or Isopropyl ß-D-1-
86 thiogalactopyranoside (IPTG) (0.1 mM).

87 **Phylogenetic analysis of FliC**

88 To investigate the structural diversity of the outer domains of flagellins and their presence/absence
89 across the tree of life, eight flagellin amino acid sequences (Table S2) were used as reference
90 sequences for a BLASTP search in Uniprot (30) (E-Threshold - 10; Matrix – BLOSUM62; Filter – None;
91 Gapped – Yes; Hits – 1000; HSPs per hit – All). All hits for each BLASTP search were selected and used
92 in ID mapping from database UniProtKB AC/ID to UniRef90 (31). The clusters were sorted by member
93 size and each list was manually curated to select 210 representative flagellin sequences for further
94 phylogenetic analysis, in addition to 14 FliD amino acid sequences as an outgroup. The 224 sequences
95 were downloaded in FASTA format and uploaded to CIPRES Science Gateway (32). Alignment of the
96 representative sequences was performed using MUSCLE (3.7) (33) and viewed using AliView (34). The
97 alignment was used to generate a phylogenetic tree using IQTree (2.3.2) (35) using CIPRES (for
98 parameters see Table S3) (32). 682 representative flagellins were downloaded from the
99 supplementary information from Fields *et al*. (20), 605 were available on the Uniprot database and
100 the amino acid sequences used in a MUSCLE alignment (33). A FastTree2 (36) phylogenetic tree was
101 generated using CIPRES (32). Phylogenetic trees were visualised and annotated in ItoL (v6) (37).

102 **Design and structural prediction of chimeric FliC**

103 To study the effect of outer domain manipulation on bacterial motility, a rational design approach for
104 each chimeric FliC candidate was taken. It was decided to maintain the D0 and D1 domains of *E. coli*
105 K-12 FliC (UniProt - P04949) (EEEE) in order to preserve the export recognition sequence found in the
106 D0 domain (12). Therefore, to understand if these outer domains are functionally required for
107 flagellum formation, the first *E. coli* K-12 *fliC* variants designed were the *fliC_ΔD3* (EEE) and *fliC_ΔD2/3*
108 *(EE)*, with deletions of residues 195-300 and 174-405, respectively. For the two-species and three-
109 species FliC chimeras, five flagellin amino acid sequences were used from across the bacterial tree of
110 life, *Helicobacter mustelae* (P50612), *Mesorhizobium* sp. ORS3359 (A0A090GIX0), *Pseudomonas*
111 *aeruginosa* (P21184), *Collimonas fungivorans* strain Ter331 (G0AIL6) and *Salmonella* Typhimurium
112 strain LT2 (P06179). The D2 and/or D3 domains of these flagellins were determined manually and used
113 to replace the D2 and D3 domains of *E. coli* FliC. In the case of *S.* Typhimurium additional chimeras
114 were made using the D2 domain of *S.* Typhimurium and the D2 domains (in place of the D3 domains)
115 of *Mesorhizobium* sp. ORS3359, *P. aeruginosa*, *C. fungivorans* strain Ter331 were used. Structural
116 prediction for all domain deleted and chimeric FliC sequences was performed using AlphaFold3
117 (v3.0.1) (38).

118 **Cloning of FliC chimeras into expression vector**

119 Reverse translation of each FliC chimera from amino acid to nucleotide codon optimised sequence for
120 *E. coli* was performed in Benchling (Benchling Inc). Gene fragments for all chimeras and outer domain
121 deleted FliC were order using TWIST (Decode Bioscience) with EcoRI and NotI restriction sites
122 incorporated in the 5' and 3' regions, respectively. Digestion of gene fragments and pET21(+) (Merck)
123 expression vector was performed using EcoRI-HF and NotI-HF (NEB), followed by Antarctic
124 phosphatase (NEB) treatment of pET21(+), as per the manufacturer's instructions. Ligation was carried
125 out using T4 ligase (NEB) as per manufacturer's instructions and incubated at room temperature
126 overnight. After heat inactivation, the ligation mix was transformed into to chemically competent *E.*
127 *coli* 10-β (NEB) as per manufacturer's instructions. Transformations were incubated at 37°C for 1 hr
128 and plates on LB plates with AMP. Colonies were screened using pET21_F and pET21_R primers and

129 Q5 Polymerase (NEB) (See Table S4 for primer details and PCR conditions). Positive colonies were
130 grown overnight in LB medium with AMP and plasmids were purified using a Qiagen Miniprep Kit,
131 following manufacturer's protocol. Purified plasmid was sent for Sanger sequencing performed by the
132 Ramaciotti Centre for Genomics (UNSW, Sydney). The confirmed plasmids were then transformed into
133 chemically competent *E. coli* SYC29 pDB108 (Δ*motAB*, *fliC*::*tetR*) and screened on LB plates containing
134 AMP, CAM and TET. This strain was selected due to its inducible motility characteristics. Positive
135 colonies were screened using the same PCR method as previously described and grown overnight in
136 LB medium containing AMP, CAM and TET.

137 **Assessing the motility of chimeric FliC expressing *E. coli***

138 Swim plate (0.30%, 0.35% and 0.40% agar) motility assays were performed with required antibiotics
139 and inducers as previously described (39). Plates were inoculated with 1 µl induced overnight culture
140 and incubated at 30°C for 16 hrs. Plates were imaged using a Bio-rad Gel Doc XR with Gel
141 documentation system with analysis of Image Lab 6.0.1 (No filter, White Epi Illumination). Swim ring
142 diameters were measured using ImageJ (FIJI) (40). Data were analysed and displayed using GraphPad
143 2.0 (version 9.5.1).

144 **Free swimming assay for motile chimeric FliC expressing *E. coli***

145 Mutant FliC expressing *E. coli* were grown overnight in LB medium containing appropriate antibiotics
146 and inducers at 30°C. A 1% inoculum was grown in TB medium until OD_{600} 0.5. A tunnel slide was
147 formed using tape and a glass cover slip and 30 µl of sample was flown through, as previously
148 described (41). Cells were visualised using a phase contract microscope (Leica) and imaged using a
149 Nikon camera. For each sample 10 videos of 400 frames (20 frames/second) were recorded.
150 Recordings were analysed in a custom programme in LabVIEW (National Instruments) (39) to quantify
151 the swim speeds of each motile construct. GraphPad 2.0 (version 9.5.1) was used to analyse and
152 display data.

153 **Flagella staining of chimeric FliC expressing *E. coli***

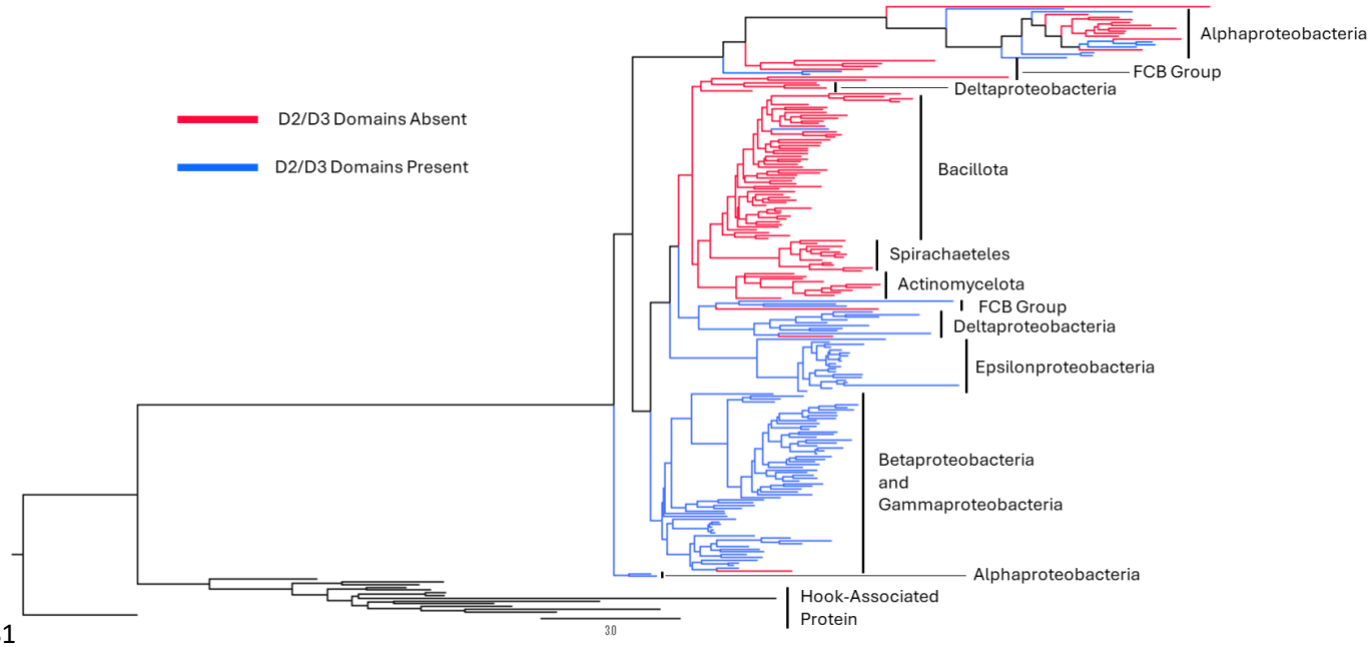
154 Staining of flagellar filaments was performed using Remel ^{TM} Flagella stain (Thermofisher) as per the
155 manufacturers protocol with minor changes. Strains of interest were inoculated on 0.3% LB agar
156 plates, with appropriate antimicrobials and inducers, and incubated for 16 hrs at 30°C. To the edge of
157 each swim ring 30 µl of LB medium was added and incubated for two mins at room temperature after
158 which 10 µl was spotted on a glass slide. After this step the protocol was followed exactly. Samples
159 were visualised using a Leica DM750 microscope at 100x magnification and images captured using a
160 MEE-500 camera (Yegren, China) and the camera app on Windows 10 (v 10.0.1.19045). Filament
161 lengths were measured using ImageJ (FIJI) (40) using the freehand line tool. Data were analysed and
162 displayed using GraphPad 2.0 (version 9.5.1).

163 **Results**

164 **Flagellins cluster according to primarily phyla**

165 To study the structural diversity of flagellin outer domains, 210 representative flagellin amino acid
166 sequences from across the bacterial tree of life were selected, aligned and used in the generation of
167 a phylogenetic tree (Figure 1). Of the representative flagellin sequences selected 53% (112/210)
168 contained the outer D2 and D3 domains. The majority of the flagellins clustered according to the phyla
169 or class to which the bacteria encoding the respective flagellin gene belonged, also seen in
170 phylogenetic analysis of 605 flagellin sequences from Fields *et al*., (20) (Figure S1A and B). In
171 *Spirochaetota* (12/12) and *Actinomycelotota* (10/10), all flagellins did not contain the outer domains

172 (Figure 1, red branches). In *Bacillota,* none of the flagellins contained outer domains, with one
173 exception (53/54). The classes *Epsilonproteobacteria* (20/20) and *Beta/Gammaproteobacteria* (66/67)
174 all clustered within their respective class and all contained the outer domains, with one exception
175 (Figure 1, blue branches). *Deltaproteobacteria* and the *Fibrobacteres-Chlorobi-Bacteroidetes* (FCB)
176 superphylum had flagellin sequences both with and without outer domains and the clading of these
177 was split on the presence or absence of the outer domains. *Alphaproteobacteria* flagellins, in contrast,
178 were the only group where sequences with and without the outer domains where mixed in the one
179 clade (10 with and 12 without, respectively). In *Deltaproteobacteria* and the FCB superphylum, 64%
180 (9/14) and 42% (5/12), respectively, contained outer domains.



181

182 **Figure 1**. Phylogeny of 210 representative flagellin sequences, with 14 flagellar hook associated
183 proteins as an outgroup. Branches are coloured based on the presence (Blue) or absence (red) of the
184 D2 and D3 domains.

185 The majority of flagellins containing outer domains were isolated from animal and water associated
186 environments (Figure S2A), whereas flagellins lacking the outer domains were found across soil,
187 animal associated and aquatic environments (Figure S2B). This was also observed when comparing
188 isolation sources, with a higher percentage of flagellins that contained outer domains were isolated
189 from animal and plant sources (63% and 55%, respectively) (Figure S2C). However, flagellins lacking
190 the outer domains were found predominantly in soil isolates (70%). There was a near equal percentage
191 of flagellins containing outer domains and flagellins with no outer domains isolated from aquatic
192 environments (51% and 49%, respectively). Within each phylum-based cluster there was a minimal
193 association of a flagellin type with a particular environment, with the exception of
194 *Epsilonproteobactera*, 88% of which were isolated from animal sources, and *Alphaproteobacteria*,
195 which were all either from aquatic or plant associated environments.

196 **Outer domain deleted and chimeric flagellin can rescue motility in *E. coli* K-12**

197 First, to investigate the potential redundancy of the outer domains for bacterial motility we deleted
198 the nucleotide sequence coding for the outer domains from *E. coli* K-12 *fliC.* The nomenclature used
199 for all flagellin outer domain deletions and chimeric variants created in this work represents each
200 domain (D0-D3) as a single character of the first letter of the genus to which the flagellin is derived,

thus wild type *E. coli* K-12 flagellin is termed EEEE. Structural prediction of these outer domain deletions indicated that sequential removal of these domains, removing D3 to generate EEE and both D2 and D3 to generate EE, would not effect the structure of the core domains (Figure 2A, Figure S3). All PAE scores indicated high confidence at the N- and C-termini with reduced confidence in the central section of the protein, correlating to the position of the outer domains (Figure S4A-N). We then designed chimeric *fliC* variants using the D0 and D1 domains of *E. coli* K-12 and the outer domains of *S.* Typhimurium (EESS/EEES), *H. mustelae* (EEH/EEEH) and *C. fungivorans* (EEC/EEEC) (Figure S3A and D). The D0 and D1 domains were maintained as they are in *E. coli* K-12 to retain the export recognition site found between residues 26-47, given our use of *E. coli* K-12 proteins for all other elements of the motor, including flagellin export (12). *S.* Typhimurium (*Gammaproteobacteria*) (EESS/EEES) and *C. fungivorans* (*Betaproteobacteria*) (EEC/EEEC) were selected as members of the *Pseudomonadota* phylum, which clustered in the same clade as *E. coli* K-12 FliC (Figure 1). The outer domains of *H. mustelae* (EEHH/EEEH), belonging to *Epsilonproteobacteria*, were selected due to being in a cluster where all members contained the outer domains (Figure 1). The structures of these chimeric FliC variants were predicted using AlphaFold3 and these structural predictions showed that the tertiary structure of the core domains were conserved (Figure 2B, Figure S3).

In addition to direct replacement of the outer domain replacement with a domain from a single species, we decided to engineer three-species FliC chimeras. This was to assess the inter-compatibility of the outermost D3 domain. The D2 domain of *S.* Typhimurium was used for all D2 domains in these three-species FliC constructs (Figure S3). The D3 domain was replaced with the outer domain from *C. fungivorans* strain Ter331 (EESC), *P. aeruginosa* (EESP), *Mesorhizobium* sp. ORS 3359 (EESM) (Figure 2C). The D3 domain from *C. fungivorans* strain Ter331 was chosen due to its FliC phylogenetic clading with the FliC from *E. coli*. Alternately, the D3 domain from *P. aeruginosa* and *Mesorhizobium* sp. ORS 3359 were chosen as previous work showed that the outer domains of *P. aeruginosa* PA01 and *Sinorhizobium meliloti* formed ridged filaments formed by interaction between the flagellin outer domains (Figure 2C) (21,22). Overall, chimeric and outer-domain-deleted FliC ranged in size from 266 to 535 residues in length, with the outer D2 and D3 domains ranging from 107 to 269 amino acids in length (Table S4). Additionally, the outermost D3 domain of the *E. coli* K-12 flagellin was replaced with the outermost domain from *S.* Typhimurium, *Mesorhizobium* sp. ORS 3359, *C. fungivorans*, *P. aeruginosa* and *H. mustelae* (Figure 2D).

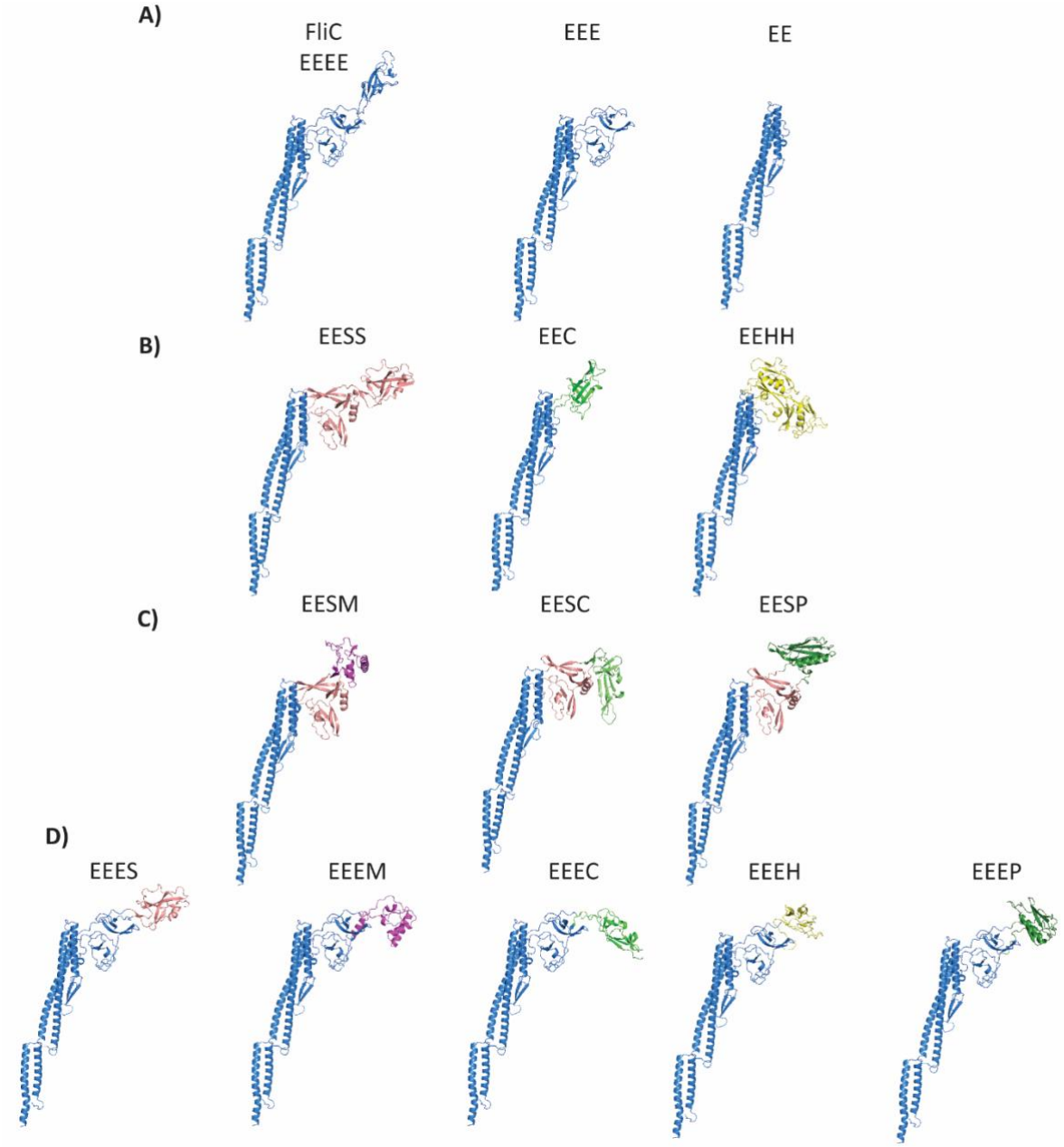


Figure 2. AlphaFold3 predictions of chimeric and outer-domain-deleted *E. coli* K-12 FliC monomers designed for this study. A) Predicted structures of wild type, D3-deleted and D2/3-deleted flagellin. B) Predicted structure of *E. coli* K-12 D0 and D1 domains and single species outer domains. C) Predicted structures of *E. coli* K-12 D0 and D1 domains and multi-species outer domains.D) Predicted structures of E. coli K-12 D0, D1 and D2 domains and single species outer domains. D) Predicted structures of *E. coli* K-12 D0, D1 and D2 domains with the D3 domain replaced with the outmost domain of a single species flagellin. *E. coli* K-12 (Blue), *H. mustelae* (Yellow), *C. fungivorans* (Light Green), *S.* Typhimurium (Pink), *Mesorhizobium* sp. OR3359 (Purple) and *P. aeruginosa* (Dark Green).

Next, we investigated whether chimeric FliC could form functional filaments and therefore rescue motility in filament-disrupted strains (*fliC::tetRA*). Plasmids expressing FliC mutants or flagellar stators were co-transformed into an *E. coli* Δ*motAB fliC::tetRA* strain. Motility of outer-domain-deleted and chimeric FliC expressing strains was confirmed using swim plate motility assays. Both outer domain deletions produced swim rings (Figure 3). The average swim ring diameter when both outer domains were deleted (EE) was larger, 14.5 ± 2.5 mm, than that with only the D3 domain deleted, 11.9 ± 2.5

mm, however this difference was not statistically significant (Figure 3). However, both these outer-domain-deleted flagellin mutants produced significantly smaller swim rings than wild type FliC. Likewise, motility of the two-species outer domain FliC mutants, EESS, EEH and EEC, was confirmed using a swim plate motility assay. Only EESS was motile, with a swim ring diameter of 9.9 ± 2.1 mm (Figure 3). EEC and EEH produced no swim rings (Figure 3). Only one of the three-species chimeras were observed to be motile. The FliC chimera that contained the D3 domain from *Mesorhizobium* sp. OR3359 consistently produced a swim ring diameter of 10.6 ± 0.3 mm, whereas EESC and EESP were not motile (Figure 3). All flagellin chimeras with the D3 domain of *E. coli* K-12 replaced by the outermost domain of another bacterial flagellin were non-motile.

As the viscosity of agar plates was increased, the swim ring diameter decreased significantly (Figure S5A), with the overall relationship between the chimeric FliC expressing strains and WT FliC remaining the same (Figure S5 B). However, when normalised to the swim ring diameters using 0.3% agar there were no significant differences in the percentage of the swim ring diameter between any motile flagellin variant and wild type at either 0.35% or 0.4% agar (Figure S5B).

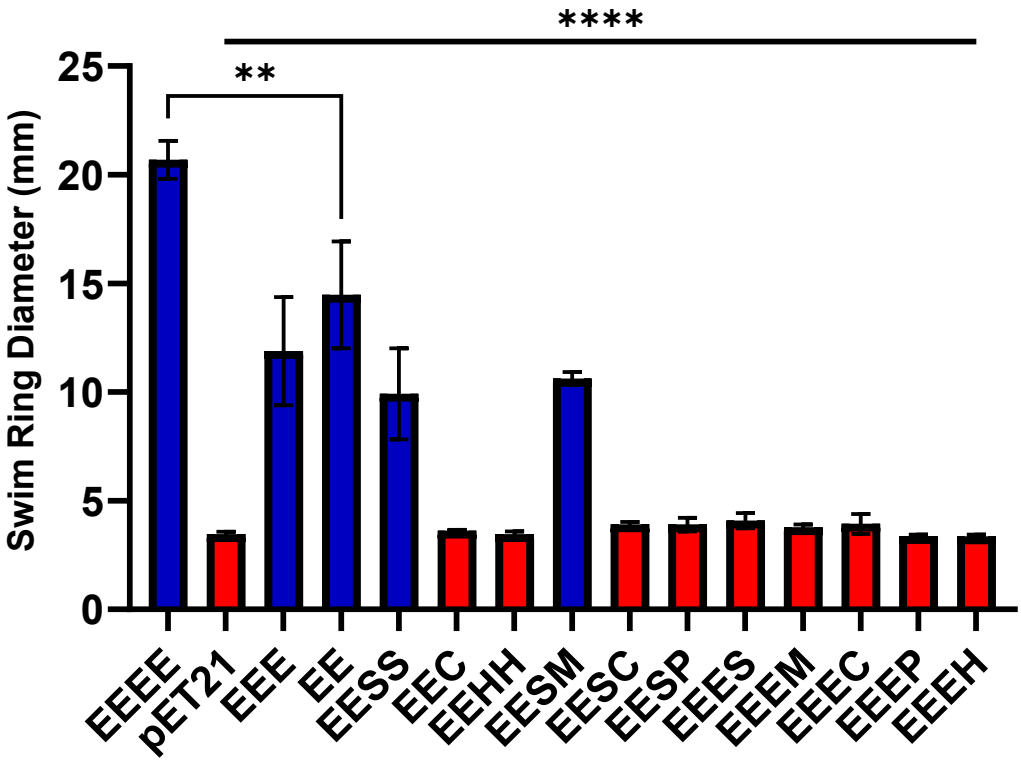
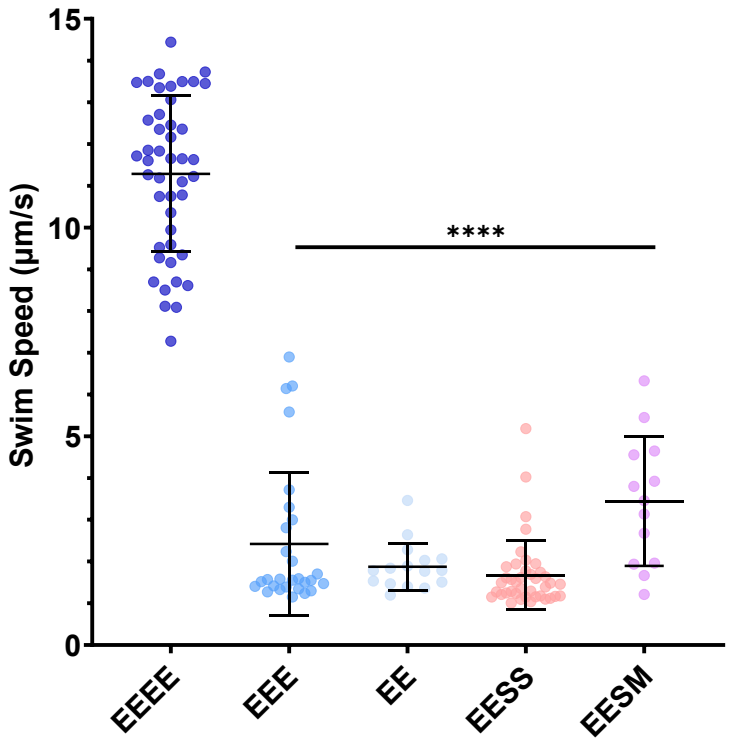


Figure 3. Mean swim ring diameter of cells expressing each chimeric and outer-domain-deleted FliC recorded from three independent 0.3% swim plates incubated at 30°C for 16 hrs. Motile and non-motile flagellin variants expressing chimeric FliC are indicated by blue and red bars, respectively. Error bars indicate standard error of the mean. A two-way ANOVA comparing each column to the FliC value was used to generate the p values (** = ≤ 0.01, *** = ≤ 0.001).

Motile FliC chimeras that produced significantly lower swim speeds

Free swimming assays were used to measure the swimming speeds for each of the motile FliC strains that were generated as part of this work. Strains expressing EEEE (11.2 ± 2.3 µm/s) had swim speeds significantly faster than the other motile variants (p value < 0.0001) (Figure 4). Strains expressing EEE

272 (2.4 ± 1.7 µm/s), EE (1.9 ± 0.6 µm/s), EESS (1.7 ± 0.8 µm/s) and EESM (3.44 ± 1.5 µm/s) had significantly
273 lower swim speeds.



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275 **Figure 4.** Free-swimming speeds for motile chimeric and truncated flagellin in µm/s. Error bars
276 indicate standard deviation. A two-way ANOVA comparing each column to EEEE was used to generate
277 the p values. Cells expressing wild-type FliC (EEEE) are significantly different to all other FliC variants(p
278 value ≤ 0.05) (**** = ≤ 0.0001).

279 **Crystal violet staining of filaments shows formation of chimeric filaments**

280 Staining of flagella confirmed export of flagellin and the formation of filaments. Filaments were
281 observed in all motile strains (Figure 5A). For all non-motile strains there were no observable
282 filaments. Filament length was determined for all motile strains with EEEE (FliC), producing the longest
283 filaments at 5.7 ± 1.6 µm (Figure 5B). The outer domain deleted contructs EEE and EE produced
284 significantly shorter filaments at 2.7 ± 0.9 µm and 1.9 ± 0.5 µm, respectively. Shorter filaments were
285 also formed in the two-species chimera EESS (2.8 ± 1.1 µm) and in the three-species chimera EESM
286 (3.4 ± 1.1 µm) (Figure 5B). All motile cells expressing domain-deleted or chimeric FliC had significantly
287 shorter filaments than cells expressing wild-type FliC (EEEE).

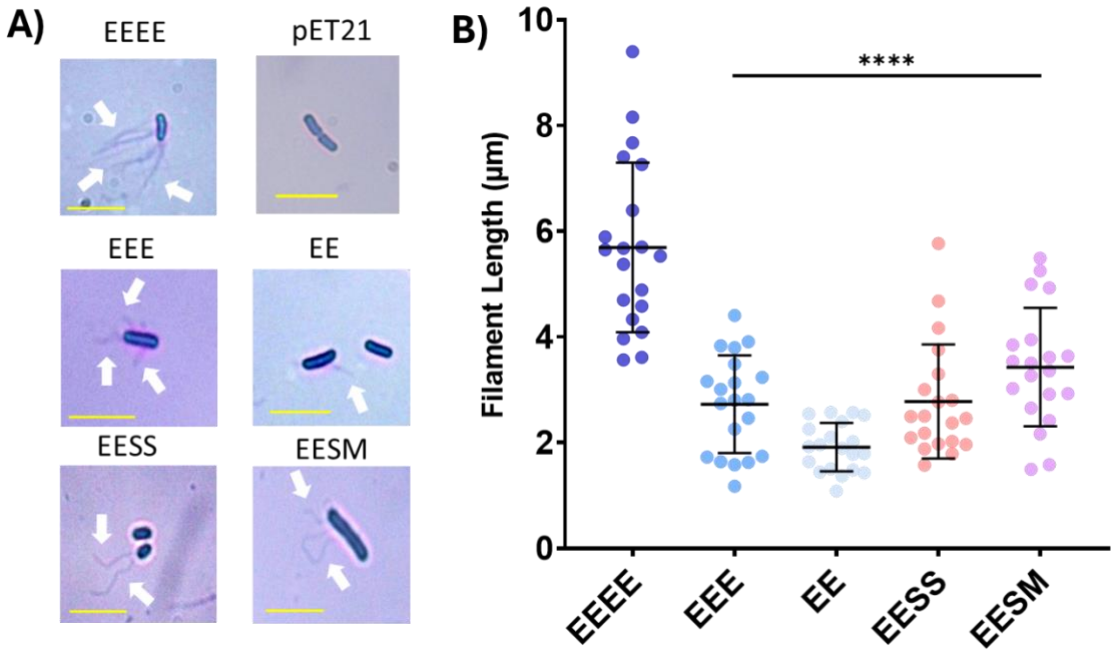

Figure 5. A) Analysis of filament export and assembly. Reprentative images of motile strains after flagella staining. The pET21 empty vector is shown as a representative non-motile variant. Scale bar (Yellow) represents 5 µm. White arrows indicate presence of flagella. B) Lengths of FliC chimeric filaments. Error bars represent standard deviation. A two-way ANOVA comparing each column to FliC was used to generate the p values (**** = ≤0.0001).

Discussion

In this study, we investigated whether expression of *E. coli* FliC with deleted or replaced outer domains (D2 and D3) could rescue motility in *fliC*-deleted strains of *E. coli*. We observed that flagellin outer domains were not required for motility in *E. coli* and that two and three-species FliC outer domain chimeras are able to form functional filaments. From our phylogenetic analysis we showed that bacteria have evolved flagellar filaments with and without outer domains. Th presence or absence of these domains is distributed in a way that correlates with bacterial clades. A greater proportion of flagellins with outer domains were isolated from animal sources. Previous work has shown that the presence of these hypervariable domains can aid in immune evasion (42). Horizontal gene transfer of complete sets of flagellar genes has been demonstrated as well as evidence of specific recombination of the flagellin outer domains (43,44,19). Here, we we constructed FliC with chimeric or deleted outer domains to show that recombination of outer domains can form functional filaments, thus providing evidence that recombination events can occur across multiple genera with compatible outer domains.

FliC outer domains can affect bacterial motility

Although originally thought that the outer domains have no interaction with the core domains or each other (7,13), recent studies have shown that in some cases there are inter-domain interactions which help to stabilise the filament to aid motility (21). In Kreutzberger *et al.*, this phenomenon was identified in *Achromobacter* filaments where the outer domains exist in four conformations and can form a tetrameric sheath which surrounds the filament (22). The authors also observed outer domain interactions in *Sinorhizobium meliloti* forming a dimeric outer domain interaction in two conformations (22). Similarly, *P. aeruginosa* PAO1 flagellin has been shown to form a ridged filament

315 due to interactions of its outer domains (21). This suggests that along with the well-studied role of
316 immune activation of the flagellin outer domains, there is also a role that these domains have on
317 bacterial swim speed in different environments (25). In *S.* Typhimurium and *E. coli*, it has been
318 previously shown that partial outer-domain-deleted FliC mutants are motile (45,46), however, full
319 D2/3 deletions were non-motile, whereas our results show that *E. coli* K-12 expressing FliC deleted for
320 domains D2 and D3 is still motile to some degree. This demonstrates that the outer domains of the *E.*
321 *coli* K-12 flagellin are not absolutely required for motility. We observed rescue in motility when
322 replacing the D2/3 domains with those from the *S*. Typhimurium flagellin as well as a two species
323 chimera with *S.* Typhimurium and *Mesorhizobiium* sp. ORS 3359. Future structural work would
324 characterise outer domain interactions in these mutant filaments quantify any effect on the
325 superstructure of the filament.

326 The lack of motility in the majority of chimeric flagellin expressing strains could also arise from the
327 lack of post-translational modifications. It is known that many flagellins, although not *E. coli* K-12,
328 require glycosylation or methylation for the formation of the flagella filament (47,48).
329 *Epsilonproteobacteria*, in which *H. mustelae* is found, require glycosylation for the formation of the
330 flagellar filament (49). Both *P. aeruginosa* a-type and b-type flagellins also are glycosylated on their
331 exposed surfaces (50). This may be the reason why no motility of some of the flagellin chimera
332 expressing strains was observed. The levels of flagellin expression may also be an issue, compounded
333 with complex flagellin structures that could potentially influence the formation of the flagellar
334 filament. Previous work showed that motility was only partially restored (~50%) when a rhamnose
335 promoter was used when compared to the wild type flagellin promoter (51). Therefore, optimisation
336 of the flagellin expression system, using various promoters and/or expression vectors, could increase
337 the levels of chimeric flagellin expression and thus increase the likelihood of the formation of the
338 flagellar filaments. Additionally, due to the use of multiple antibiotics to select and maintain the two
339 expression plasmids there may have been a reduction in flagellin expression and therefore a reduced
340 likelihood of functional filament formation (52).

341 Bacterial swim speed can vary widely with *E. coli* swim speeds ranging from 14.2-28.8 µm/s,
342 *Bdellovibrio bacteriovorus* at 100 µm/s and *Thiovulum majus* at approximately 600 µm/s (53–56). *E.*
343 *coli* and *T. majus* both contain flagellin outer domains whereas the *B. bacteriovorus* flagellin does not.
344 We observed lower swim speeds and shorter filaments for EESS, EESM, EEE and EE expressing bacteria.
345 Previous work has shown that even a single polymorphism (at position N87 in *E. coli* K-12 FliC)
346 prevented motility in structured environments, although motility was observed in liquids (29).
347 Therefore, it is plausible that whole domain replacements may alter the polymorphic transformation
348 of the flagellar filament which allows for raised observable motility within soft agar. Additionally,
349 reductions in motility may arise due to weaker filaments being more prone to shearing or due to
350 differences in the filament export rate.

351 **Outer domain chimeras require *S.* Typhimurium D2 domain to form filaments**

352 We observed that two out of eleven FliC variants rescued motility, with the remainder of the chimeras
353 being non-motile. In work by Nedeljkocić *et al*., 13 flagellin chimeras, with combinations of all
354 domains, were generated using the FliC from *S.* Typhimurium and *P. aeruginosa* PAO1 and only one
355 multi-genus chimera produced a functional filament (21). Furthermore, in the same study the authors
356 generated flagellin chimeras using domain swaps of two *P. aeruginosa* strains (PAO1 and PAK), where
357 only two variants (where the D0 domain was exchanged) were motile (21). This further illustrates that
358 the production of flagellin chimeras even within the same bacterial species does not yield a high
359 proportion of motile variants. In *E. coli* SYC29 expressing chimeric-FliC containing outer domains,
360 motility was only rescued only in chimeras that contained the *S.* Typhimurium D2 domain. This may

361 indicate that there are key structural elements or residues found in this domain that allow of export
362 and formation of a functional filament.

363 **Conclusion**

364 Engineering of the flagellin outer domains can not only increase our understanding of filament
365 formation but could also provide biotechnological benefits. Flagellin is already used to display antigens
366 from other organisms and stimulate the immune system and has been engineered to contain
367 fluorescent and enzymatic domains (25,57,58). Here we sought to develop chimeras for the outer
368 domains of *E. coli* K-12 flagellin to provide evidence that recombination events have the potential to
369 produce functional filaments. We have shown that two- and three-species chimeras can form
370 functional filaments. However, our work illustrates the challenges of exchanging whole flagellin outer
371 domains between bacteria. Further analysis is required to fully understand the export, folding and
372 incorporation of chimeric flagellins and how they interact with other proteins within the bacterial
373 flagellar motor. Future work will investigate how hypervariable FliC domains can affect motility in
374 response to environmental changes and establish FliC and its homologs as a synthetic biology platform
375 for biotechnological applications.

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392 **Conflict of Interest**

393 The authors declare no conflict of interest.

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