Manuscript Number: COMICR-D-15-00068R1

Title: Motility in the epsilon-proteobacteria

Article Type: SI: 28 Growth&Devel:Eukar/Prokar 2015

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Abstract: The epsilon-proteobacteria are a widespread group of flagellated bacteria frequently associated with either animal digestive tracts or hydrothermal vents, with well-studied examples in the human pathogens of *Helicobacter* and *Campylobacter* genera. Flagellated motility is important to both pathogens and hydrothermal vent members, and a number of curious differences between the epsilon-proteobacterial and enteric bacterial motility paradigms make them worthy of further study. The epsilon-proteobacteria have evolved to swim at high speed and through viscous media that immobilize enterics, a phenotype that may be accounted for by the molecular architecture of the unusually large epsilon-proteobacterial flagellar motor. This review summarizes what is known about epsilon-proteobacterial motility and focuses on a number of recent discoveries that rationalize the differences with enteric flagellar motility.

1 Motility in the epsilon-proteobacteria

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8 Abstract

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18 Introduction

19 Motile epsilon-proteobacteria are found in diverse habitats, most commonly animal digestive tracts or
20 hydrothermal vents (Figure 1). Best studied are the animal digestive tract-associated species [1], prominently
21 the human gastrointestinal pathogens *Campylobacter jejuni* and *Helicobacter pylori*. *C. jejuni* and
22 related species are leading causes of gastroenteritis, yet often harmless commensals of birds [2]. Other
23 members of order *Campylobacterales* are also associated with mammalian intestinal tracts,
24 including *Wolinella succinogenes*, a cattle rumen commensal [3], and pathogenic *Arcobacter* species [4].
25 *Helicobacter* species are invariably digestive tract-associated, with *H. pylori* colonizing 50% of humans on
26 the planet and although associated with gastritis, gastric ulcers and gastric carcinoma, is also associated with
27 beneficial outcomes with gastroesophageal reflux and asthma, indicating that the relationship between
28 human host and *H. pylori* are more complex than first thought [5–7]. Similar observations of pathogenesis
29 have been made for *H. mustelae* in the ferret gastrointestinal tract and *H. felis* in mice [8], and *Helicobacter*
30 *hepaticus* causes chronic hepatitis, liver cancer and inflammatory bowel disease also in mice [9,10]. Yet
31 despite numerous examples of digestive tract-associated epsilon-proteobacteria, there are also many
32 environmental species. Many are chemolithotrophs [11] that obtain energy by oxidizing compounds found in
33 their environment. The *Sulfurospirillum*, *Sulfurimonas*, *Sulfuricurvum*, *Thiovulum*, and some *Arcobacter* genera
34 from the *Campylobacterales* order are environmental bacteria [12,13], with habitats ranging from marine

35 hydrothermal vents and coastal sediments [14], underground oil-storage facilities [15], plant roots in salt
36 marsh sediments [16] and pond mud [17]. Intriguingly, there are multiple cases of epsilon-proteobacteria that
37 combine animal association with hydrothermal vent environments by establishing symbioses with gastropods
38 and annelids endemic to vents [18,19].

39 Flagellar motility is important to both pathogenic [2] and hydrothermal vent-associated [20] species. In
40 animal-associated species, motility is critical for host colonization and virulence by enabling traversal
41 through viscous gastrointestinal mucous [2,21]. Motility is also clearly important for environmental members
42 of the epsilon-proteobacteria as demonstrated by high expression levels of flagellar genes in hydrothermal
43 vent epsilon-proteobacteria [20,22]. In environmental *Thiovulum* spp., flagella play key roles in nutrient
44 acquisition whereby cells attach to surfaces and rotate their flagella to increase oxygen and sulphide flux for
45 metabolism [23].

46 Despite using homologous flagellar systems, there are striking differences between epsilon-proteobacterial
47 and the enteric motility models *Escherichia coli* and *Salmonella enterica* s.v. Typhimurium. While enterics
48 are often peritrichous (multiple flagella distributed over the cell body), the epsilon-proteobacteria have one or
49 a few polar flagella. And yet despite generally having fewer flagella, representative epsilon-proteobacteria
50 swim faster than the enterics in low-viscosity media, and continue to swim at high speed even in high-
51 viscosity environments that immobilize the enterics [24–26]. This review highlights the unique features of
52 epsilon-proteobacterial motility that may play a role in their unique range of habitats.

53 **Epsilon-proteobacterial swimming ability**

54 Characteristic of the epsilon-proteobacteria is their ability to swim rapidly, and to continue swimming even
55 in high viscosity environments. *C. jejuni* [24] and *H. pylori* [26] are capable of swimming in viscous fluids
56 that immobilize *E. coli* faster even than *E. coli*'s fastest swimming speed. A number of observations suggest
57 that this characteristic swimming may be a result of higher torque production than enteric bacteria. The
58 torque of epsilon-proteobacterial flagellar swimming has been measured for *H. pylori*, yielding an estimate of
59 3600 pN nm [27], approximately three times that observed in *E. coli*, of 1260 pN nm [28,29]. Observations
60 of *Helicobacter felis* shows swimming behavior capable of propelling the bacterium between tissues [30],
61 and *Thiovulum majus*, despite being a giant ~9 µm-diameter cell, is the second fastest-swimming bacterium

studied to-date, capable of swimming at over 600 $\mu\text{m/s}$ [25]. It is also thought that high torque and the large cell size contributes to self-organization of *Thiovulum* cells into 2D hexagonal lattices on surfaces which may contribute to increased nutrient flux [23]. Although other epsilon-proteobacteria have not been studied in similar detail, their ability to swimming through viscous media, and prevalent observations of rapid, darting motility, suggest similar mechanisms underly the characteristic swimming ability of all epsilon-proteobacteria (Figure 1).

Cell biology associated with motility

Epsilon-proteobacteria are often slender spiral rods with polar flagella (Figure 1). *Campylobacter* species tend to be approximately 0.5 μm wide and over 1 μm long, while *Helicobacter* species can be up to 1 μm wide and correspondingly longer. Although it has been suggested that *C. jejuni*'s swimming ability is partially a product of using its spiral shape for traction [31], rod-shaped *H. pylori* mutants swimming ability is unimpaired in both low and high viscosity media [32], suggesting that spiral shape may not be directly involved in motility. Deeper-branching epsilon proteobacteria *Nautilia* and *Nitratiruptor* are not spiral-shaped, but also have polar flagella [11,33]. Furthermore, *Thiovulum* spp. are also extremely fast swimmers despite being very large (~9 μm) ovoids with peritrichous flagella. Together these observations indicate that while helical cell shape may be involved in epsilon-proteobacterial motility, it is not an essential determinant.

Beside the possible contribution of cell shape to motility, *Helicobacter* species use other strategies that may enhance motility. *H. pylori* colonizes the acidic mucous of the stomach, where acidity makes the mucous form a gel [27]. To survive and maintain motility, *H. pylori* secretes a urease which breaks down urea to form ammonium, raising local pH, and reverting mucous to a viscous fluid. Some *Helicobacter* species such as *Helicobacter muridarum* [34] and *Helicobacter trogonum* [35] also assemble distinctive helical periplasmic non-flagellar ribs within their periplasm. The significance of these ribs is unclear, but may further augment the spiral characteristics of these species, or reinforce cell shape in bacteria which may be exposed to large torques that might otherwise buckle or misshape the cell.

Correct cellular localization of flagella is important for full motility of the epsilon-proteobacteria. Flagellar localization and number are controlled by a GTPase, FlhF, and putative ATPase, FlhG, respectively [36]. Although the mechanism behind FlhF and FlhG function is unclear, they likely dictate the variations across

89 the epsilon-proteobacteria (Figure 1), from a single motor at one pole (monotrichous), a single motor at each
90 pole (amphitrichous), multiple motors at one pole (lophotrichous), to multiple motors at each pole.
91 Exceptions to polar localization are seen in *Helicobacter mustelae* [37], with occasional lateral flagella, and
92 the hundreds of peritrichous flagella in *Thiovulum* spp. [38]. Electron cryo-tomography studies of *C.*
93 *jejuni* pole morphology reveal a ribosome exclusion zone at flagellated poles [39] and a single polyphosphate
94 storage granule beneath the flagellar motor. The significance of the ribosome exclusion zone and the storage
95 granule are unclear, but they may be involved in aiding flagellar rotation.

96 **Epsilon-proteobacterial flagella**

97 The machine that drives motility in the epsilon-proteobacteria is from the same widespread family of
98 machinery responsible for motility in two-thirds of bacteria: the bacterial flagellar motor [29,40,41].
99 Flagellar motors drive rotation of a flexible ~50 nm-long hook that in turn rotates a multi-micron filament to
100 coil to form a helical propeller. Flagellar motors consists of a ~50 nm-diameter cytoplasmic rotor, termed
101 the C-ring, that is rotated by a peptidoglycan-anchored stator ring. The stator is composed of a number of
102 MotA₄B₂ stator complexes that couple proton flux to rotation of the C-ring, the mechanism for which is
103 likely conserved across bacteria [42]. The C-ring is attached to the rod, an axial driveshaft that traverses the
104 periplasm, peptidoglycan and outer membrane where it attaches to the base of the hook. 'Navigation' is
105 achieved in the epsilon-proteobacteria using the same chemoreceptor system as found in other bacteria, with
106 minor variations; these are reviewed in reference [2].

107 As with peritrichous bacteria, epsilon-proteobacterial flagellar filaments are multimicron-long polymers of
108 flagellin. Some epsilon-proteobacteria, notably the *Helicobacter* spp. envelope their flagella with outer-
109 membrane extensions called sheaths. In *C. jejuni* and *H. pylori*, two flagellins, FlaA and FlaB, form the
110 filament. The major flagellin FlaA is essential, but the role of FlaB varies. Both flagellins have a divergent
111 domain D1 compared to that found in *Salmonella*; D1 is involved in polymerization of the filament and D1
112 interactions are possibly substituted by O-linked glycosylation in epsilon-proteobacteria [2]. Curiously, an
113 outcome of this is that although the *Salmonella* flagellin polymerizes into a filament composed of 11
114 protofilaments, *C. jejuni* FlaA polymerizes into a seven-protofilament filament [43].

115 How can the characteristic swimming abilities of the epsilon-proteobacteria be accounted for? It is becoming

clear that the epsilon-proteobacterial motor is considerably more complex than the enteric paradigm (Figure 2). Previous studies have sporadically reported disk-like structures beneath the outer membrane of many epsilon-proteobacteria such as *C. fetus* [44], *C. rectus* [45], *W. succinogenes* [46], and environmental *Arcobacter* species [47], observations supported by purification of disks [48,49]. Recent work in *C. jejuni* has shown that the cell pole itself has a polar depression into which the hook attaches [39,40,50]. Electron cryotomography has revealed that the epsilon-proteobacterial motor is larger than the enteric motor, with a wider C-ring and multiple additional 'disk complex' components in the periplasm [40,51]. The wider C-ring is conserved across all epsilon-proteobacteria imaged to date. *C. jejuni* and *H. hepaticus* exhibit wider C-rings with diameter 53 nm [40], while the *W. succinogenes* C-ring appears approximately ~60 nm wide in thin-section EM [48]. Inspection of this wider C-ring reveals an inner lobe that is elongated relative to that in *Salmonella*, suggestive of an additional domain or protein responsible for “spacing” the C-ring wider [51]. How do the epsilon-proteobacterial stator complexes maintain interaction with a C-ring that is wider than the enteric motor? In *Salmonella*, approximately 11 stator complexes interact with the peptidoglycan layer and P-ring [28,52,53], but *C. jejuni* has 17 stator complexes positioned at considerably wider diameter. This is also evident in both *Helicobacter muridarum* and *Helicobacter felis*, in which freeze-fracture studies revealed approximately 17 'studs' [30,34] previously shown to be stator complexes [54]. Thus in the epsilon-proteobacteria imaged so far, additional stator complexes are positioned at a wider radius than in the enteric motor.

Taken together, the combination of a wider rotor ring and additional stator complexes presents a structural rationale for the higher torque and characteristic motility of the epsilon-proteobacteria. Experiments in *E. coli* have shown that incorporation of additional stator components contribute force approximately independently to the torque of a motor [28]. With additional stators, it follows that the epsilon-proteobacterial motor must exert higher torque than the enteric motor. Furthermore, if the point of application of this force to the axial driveshaft is from a greater radius (as it is in the epsilon-proteobacteria), the torque exerted by each stator complex will further scale proportionally, resulting in a high-torque motor.

What is the role of the additional disks that form the epsilon-proteobacterial disk complex? Three disks are evident in the disk complex; the largest is a ~100 nm-diameter basal disk immediately beneath the outer membrane [10,40] attached to the conserved motor core, with two smaller disks closer to the inner

membrane named the proximal and medial disks. Because the disk complex is required for stator complex incorporation into the motor it may be that its primary role is to act as a scaffold for correct positioning of the stator complexes upon assembly into the motor to enable continued stator:rotor interactions with a wider C-ring. The significance of disk concavity in *C. jejuni* remains unclear, particularly given that this characteristic is absent from *Helicobacter* spp. disks.

Disk complex components of the *C. jejuni* motor have been identified using transposon screens [51,55,56]. Using biochemical studies of each gene in combination with electron cryo-tomography, four proteins have been located within the *C. jejuni* motor, *flgP*, *flgQ*, *pflA* and *pflB*. Abundance, outer membrane localization, and imaging reveal that FlgP forms the basal disk, likely in complex with FlgQ. PflB forms an intermediary ring around the rod, while PflA forms a wider ring above the outer membrane, and analysis of the location of the stators revealed that the stator complexes bind to the PflA ring. Five other proteins are additional candidates for further components of the *C. jejuni* motor [55]. FlgV, a transmembrane protein and CJJ81176_0996, are implicated in C-ring interaction, FlgW and FlgY, are implicated in flagellar type III secretion system interaction, and, FlgX is implicated in additional stator complex interactions. The structural roles of these proteins remain to be confirmed.

Conclusions

Epsilon-proteobacteria swim rapidly and through high-viscosity environments. A number of factors may contribute to this characteristic motility, and recent studies suggested that a major factor is flagellar motors that produce higher torque than enteric bacteria. A number of key future questions will be important in the future to better understand epsilon-proteobacterial motility.

What was the selective benefit of swimming rapidly through viscous fluids that lead to evolution of epsilon-proteobacterial motility? Although convenient to cite the benefits of burrowing through viscous mucous for pathogens, the prevalence of rapid motility across both environmental and pathogenic epsilon-proteobacteria suggests an ancient, pre-pathogenesis origin. Experiments aimed at understanding how environmental epsilon-proteobacteria benefit from rapid motility may shed light on these questions. This raises another question, however: what was the selective benefit of a high-torque motor in ancestral epsilon-proteobacteria, and contemporary environmental strains? The presence of epsilon-proteobacteria in oilfields [57,58] may be

171 assisted by ability to swim through viscosities corresponding to Class A heavy oil (10 – 100 cP) which they
172 biodegrade to form more viscous forms of oil. More information on the range of environments inhabited by
173 epsilonproteobacteria may provide more data to understand these selective pressures, in turn contributing to
174 understanding how the complex epsilonproteobacterialflagellar motor evolved.

175 Finally, little is known about the biophysics of the epsilonproteobacterialflagellar motor. Further work is
176 necessary to accurately determine the torque of individual motors at high-loads in different species: does it
177 consistently produce higher torque than the enteric motor? If not, how can we explain this in the context of a
178 wider C-ring, additional stators, and standard proton motive force [59]? In *E. coli*, rotation of the flagellar
179 motor is composed of 26 steps. With a wider C-ring, does the epsilon-proteobacterial motor also rotate with
180 correspondingly more steps? Such insights may provide crucial additional information towards
181 understanding universal mechanistic details of torque generation.

182 In sum these questions will add to our understanding of epsilonproteobacteria motility, but also fundamentals
183 of the mechanics of the bacterial flagellar motor, and molecular evolution.

184 Acknowledgements

185 This work was supported by BBSRC grant BB/L023091/1.

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189 **Figure legends**

190 **Figure 1: An overview of epsilon-proteobacterial cell plan and motility.** Notes on motility are described
191 where known. References are listed in *Bibliography*.

192 **Figure 2: Imaging the epsilon-proteobacterial flagellar motor.** (a) TEM images of epsilon-
193 proteobacterial flagellar motors for *H. mustelae*[37], *C. coli* [50], *C. fetus*(left) [49], (right) [50], (b) freeze-
194 fracture images reveal 17 'studs' likely to be stator complexes in *H. felis*[30]and *H. muridarum*[34]. (c)
195 computational slices through subtomogram averages of *S. enterica*(for comparison), *H. hepaticus*, and *C.*
196 *jejuni* [40,51]. Cross-section of *C. jejuni* motor is through plane designated by white dashes. Boxes in panel
197 (c) are 100nm x 100nm. (d) schematic highlighting the differences between the *Salmonella* and *C.*
198 *jejuni*flagellar motor, with novel epsilon-proteobacteria components labelled [51].

Highlights for Beeby, "Motility in the epsilon-proteobacteria"

- Epsilon-proteobacteria inhabit animal digestive tracts or environmental niches.
- Characteristic of the group is distinctive rapid motility even at high viscosities.
- Motility is achieved by polar flagella considerably more complex than model systems.
- Studies are now identifying novel components of these complex flagella.
- Evidence suggests that motor complexity may contribute to high swimming torque.

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28th August 2015

Morgan Beeby, PhD
Lecturer in Structural Biology

Dear Editors,

Please find attached a resubmitted manuscript for the special edition of *Current Opinion in Microbiology* titled "Growth and Development: Eukaryotes / Prokaryotes".

Please note that the manuscript makes reference to unpublished work from my lab that I plan to have published by the time of this review. The review may need minor alteration to reflect this.

Yours sincerely,

A handwritten signature in dark ink, appearing to read 'M.D. Beeby', with a stylized, cursive script.

Morgan Beeby
Imperial College London, UK

Taxonomy		Species	Sneath?	Cell plan	Habitat	Notes on motility	Reference
ε-proteobacteria	Campylobacteraceae	<i>Arcobacter nitrofigilis</i>	N	0.2-0.9 μm x 1-3 μm	Salt marsh sediments associated with plant roots	Swims with rapid corkscrew motion	McClung (1983)
		<i>Arcobacter butzleri</i>	N	0.2-0.4 μm x 1-3 μm	Gastrointestinal pathogen	Rapid darting motility	Kiehlbauch (1991)
		<i>Campylobacter jejuni</i>	N	0.5 μm x 4 μm	Gastrointestinal pathogen; commensal of birds	Swims with rapid corkscrew motion. Ability to swim through viscous media.	Ferrero (1988)
		<i>Campylobacter coli</i>	N	0.5 μm x 4 μm	Gastrointestinal pathogen; commensal of birds		Yamamoto (2012)
		<i>Campylobacter fetus</i>	N	0.5 μm x 4 μm	Reproductive tract pathogen in animals		Ferris (1984)
		<i>Sulfurospirillum deleyianum</i>	N	0.3 - 0.5 μm x 1-3 μm	Anoxic mud from a forest pond in Germany		Sikorski (2010)
	Helicobacteraceae	<i>Helicobacter felis</i>	Y	0.4 μm x 5-7 μm	Cat intestine	Rapid corkscrew motility in mucous; also 'drilling' action between and into tissue cells.	Lee (1988)
		<i>Helicobacter hepaticus</i>	Y	0.2 - 0.3 μm x 1.5 - 5 μm	Livers of mice with chronic hepatitis. Human pathogen?		Fox (1994)
		<i>Helicobacter mustelae</i>	Y	0.5 μm x 2 μm	Natural inhabitant of ferret stomach. Frequently seen intracellularly.	Unusual spinning motility due to rod shape and lateral flagella? Superior swimming in viscous media to <i>E. coli</i> .	O'Rourke (1992)
		<i>Helicobacter pylori</i>	Y	0.5 μm x 2.5 - 5 μm	Animal gastro-intestinal tracts, causing gastritis. Often asymptomatic	High torque and swimming activity. 3500 pN nm estimated swimming torque.	Marshall (1985)
		<i>Sulfurimonas gotlandica</i>	N	0.5 - 1 μm x 3 μm	Mud from marine tidal flats in The Netherlands		Grote (2012)
		<i>Sulfuricurvum kujiense</i>	N	0.4 μm x 1 - 2 μm	Underground crude-oil storage facility in Japan		Kodama (2004)
		<i>Thiovulum majus</i>	N	9 - 17 μm	Black mud from marsh	Capable of swimming at ~600 μm / second. Very many peritrichous flagella.	Petroff (2015)
		<i>Wolinella succinogenes</i>	N	0.5 - 1 μm x 2 - 6 μm	Bovine gastrointestinal tract	Rapid, darting high motility	Baar (2003)
		<i>Nautilia profundicola</i>	N	0.3 μm x 0.4 μm	Deep-sea hydrothermal vent epibiont of a polychaete worm		Smith (2008)
		<i>Nitratiruptor tergacus</i>	N	0.8 μm x 2.5 μm	Hydrothermal field, Mid-Okinawa Trough, Japan	Flagellated but non-motile under culture conditions.	Nakagawa (2007)
γ-proteobacteria		<i>Salmonella enterica; Escherichia coli</i>	N	1 μm x 3 μm	Varied; gastrointestinal tract	"Normal" models for flagellated motility; estimated 1260 pN nm swimming torque.	Minamino (2008), Sowa (2008)

a Figure 2

H. mustelae

C. coli

H. felis

Salmonella

H. hepaticus

d

Salmonella

C. fetus

C. fetus

H. muridarum

C. jejuni

C. jejuni cross-section

C. jejuni

Salmonella enterica serovar Typhimurium

Campylobacter jejuni

Graphical Abstract (for review)

28th August 2015

Morgan Beeby, PhD
Lecturer in Structural Biology

Dear Editors,

Because responses to reviews were extremely minor I have made the requested alterations to the text to enhance clarity, but do not include here any specific responses to points raised.

Yours sincerely,

A handwritten signature in dark ink, appearing to read 'M.D. Beeby', with a stylized, cursive script.

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