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The flagellum in bacterial pathogens: for motility and a whole lot more

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Keywords – bacterial flagella, motility, pathogenesis, adhesion molecule, Type III secretion system, near surface swimming

Highlights

- Flagella have multiple critical roles in bacterial pathogenesis.
- Flagella-mediated chemotaxis-directed motility is critical to reach the site of pathogenesis.
- Post-motility, flagella also play many other key roles in pathogenesis.
- Examples include mechanosensory response, adhesion, biofilm formation, and secretion.
- Bacteria have also developed different mechanisms to cope with flagella being potent antigens.

Abbreviations – type III secretion system (T3SS), enterohemorrhagic *Escherichia coli* (EHEC), *Salomonella enterica* subspecies 1 serovar Typhimurium (*S.* Typhimurium), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), pattern-recognition receptors (PRRs), pathogen-associated molecular patterns (PAMPs), Toll-like receptors (TLRs), Nod-like receptor (NLR), uropathogenic *E. coli* (UPEC), intracellular bacterial communities (IBCs)

Abstract

The bacterial flagellum is an amazingly complex molecular machine with a diversity of roles in pathogenesis including reaching the optimal host site, colonization or invasion, maintenance at the infection site, and post-infection dispersal. Multi-megadalton flagellar motors self-assemble across the cell wall to form a reversible rotary motor that spins a helical propeller – the flagellum itself – to drive the motility of diverse bacterial pathogens. The flagellar motor responds to the chemoreceptor system to redirect swimming toward beneficial environments, thus enabling flagellated pathogens to seek out their site of infection. At their target site, additional roles of surface swimming and mechanosensing are mediated by flagella to trigger pathogenesis. Yet while these motility-related functions have long been recognized as virulence factors in bacteria, many bacteria have capitalized upon flagellar structure and function by adapting it to roles in other stages of the infection process. Once at their target site, the flagellum can assist adherence to surfaces, differentiation into biofilms, secretion of effector molecules, further penetration through tissue structures, or in activating phagocytosis to gain entry into eukaryotic cells. Next, upon onset of infection, flagellar expression must be adapted to deal with the host's immune system defenses, either by reduced or altered expression or by flagellar structural modification. Finally, after a successful growth phase on or inside a host, dispersal to new infection sites is often flagellar motility-mediated. Examining examples of all these processes from different bacterial pathogens, it quickly becomes clear that the flagellum is involved in bacterial pathogenesis for motility and a whole lot more.

Graphical abstract

1.0 Introduction

1.1 Motility, flagella, and pathogenesis

Successful pathogens combine a variety of capabilities that allow entry and replication within a host, while subverting or evading host defenses (Cross, 2008). A huge advantage to this end is for a bacterium to be motile - to have the ability to direct its own movement. Bacterial motility comes in a range of forms, including swimming, swarming, gliding, twitching or floating, and is generated or augmented by surface appendages such as flagella that rotate, pili that pull, 'leg-like' appendages that 'walk' and internal structures that contort (Jarrell and McBride, 2008). One of the most widespread motility machines in bacteria is the bacterial flagellum, a helical propeller that is rotated by a reversible rotary motor to confer swimming motility to cells (Chen et al., 2011; Jarrell and McBride, 2008). Flagellated motility is essential for full pathogenesis by many bacteria, including but not limited to, *Escherichia coli*, *Salmonella* spp., *Bordetella* spp., *Vibrio cholerae*, *Helicobacter* spp., *Campylobacter jejuni*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Borrelia burgdorferi* and *Treponema* spp (Josenhans and Suerbaum, 2002). And yet while the flagellum was initially thought to contribute to virulence solely as a motility device, recent research has revealed that flagella play central roles in many other infection processes such as adhesion, biofilm formation, effector molecule secretion and immune system modulation (Duan et al., 2013). This review highlights some of these disparate roles played by bacterial flagella during pathogenesis.

1.2 The bacterial flagellum – structure, assembly and function

Bacteria contain many macromolecular machines that carry out metabolic and cellular processes, maintain cell integrity and generate energy, and few of which are so striking or complex as the bacterial flagellum (Saier, 2013). Composed of around 30 unique structural proteins, ranging in copy number from a few to tens of thousands, the complete flagellar structure can measure up to 60 nm across, 10 µm long and weigh approximately 1 billion Da (Chen et al., 2011; Morimoto and Minamino, 2014; Saier, 2013). For the interested reader, there are numerous recent reviews which examine the flagellar structure and assembly process in detail (Altegoer et al., 2014; Minamino and Imada, 2015; Morimoto and Minamino, 2014; Zhao et al., 2014).

The flagellar structure is usually described in three parts: the basal body (which contains the reversible motor that anchors the structure to the membrane), the hook (which extends out from the top of the basal body and acts as a universal joint) and the filament (which extends many cell body lengths from the hook and, when rotated, forms the helical propeller) (**Figure 1**). Flagellar self-assembly is a multi-stage hierarchical process that starts with coordinated assembly of the flagellar type III secretion system (T3SS) (homologous to the T3SS core of the needle-like injectisome structure (Abby and Rocha, 2012; Egan et al., 2014)), the MS-ring in the cytoplasmic membrane and the C-ring at its cytoplasmic face (Li and Sourjik, 2011, Morimoto et al., 2014). The MS- and C-rings begin by forming a scaffold for the assembly of the cytoplasmic components of the flagellar T3SS (Abrusci et al., 2013; Hu et al., 2015). The peptidoglycan-spanning P-ring and lipopolysaccharide-spanning L-ring (in Gram-negative bacteria) assemble in association with the T3SS, providing channels through which the axial components of the flagellum can assemble and rotate. The active flagellar T3SS then recruits, unfolds, and exports proteins through the hollow core of the growing axial structure to assemble the periplasm-

spanning rod, the flagellar hook, and the flagellar filament at the distal tip in precisely coordinated order (Minamino, 2014). While the rod and hook are of determinate lengths, the filament extends to multiple microns in length.

The basal body of the flagellum includes the motor that powers rotation. Transmembrane protein complexes, known as stator complexes, transduce energy from the flow of ions (either protons or sodium) across the inner membrane to induce conformational changes that exert torque on the cytoplasmic C-ring, which is in turn coupled to the rod, hook, and filament. The propulsive force generated by this rotation results in swimming at a range of speeds, from 25-35 µm/s for *Escherichia coli* (Lowe et al., 1987) to 160 µm/s for *Bdellovibrio bacteriovorus* (Lambert et al., 2006). The range of speeds is most likely based on many factors, including cell shape (Young, 2006), motor energy source (Asai et al., 2003) and a widespread structural diversity in flagellar motors across the bacteria (Chen et al., 2011). The balance between motor torque and speed has been studied in several systems and appears to be optimized for higher power or greater efficiency, based on the cell's energetics (Chen and Berg, 2000; Li and Tang, 2006; Sowa et al., 2003).

1.3 The flagellum plays roles throughout infection

Although the specifics vary between pathogens, flagella are involved throughout the infection cycle. The pathogenic cycle can be broken down into four stages: reaching the host/target site; colonization or invasion; growth and maintenance; and dispersal to new hosts. Flagella play roles at every step in a diversity of pathogens, either by facilitating motility or fulfilling other roles. Each of these stages are discussed in detail below and additional information and examples can be found in the literature (Duan et al., 2013; Guerry, 2007;

Josenhans and Suerbaum, 2002; Moens and Vanderleyden, 1996). The widespread occurrence of flagella across all bacteria (including the majority of environmental species (Chen et al., 2011)), the disparity of roles played between different pathogens, and the likely pre-dating of flagella relative to the emergence of eukaryotes, combine to suggest that flagella are not pathogenesis organelles *per se*, but rather have been co-opted to assist the needs of various pathogens in numerous ways to enable full colonization of specific pathogenic environmental niches. This co-option can therefore be seen as one facet of the adaptive radiation of this fascinating molecular machine.

2.0 Flagella enable bacteria to swim to the host/target site

For pathogenesis, a bacterium must first find a site for infection, a task greatly facilitated by flagellated motility. In three dimensional space, a bacterium is very small compared to many of the hosts or external environments it finds itself in, making a diffusion-based search far from optimal. The first advantage that flagellated bacteria have over aflagellate bacteria is their ability to actively search their environment instead of relying on Brownian motion. Moreover, bacteria have evolved chemoreceptor systems in conjunction with their flagella to sense their environment and move in favourable directions (chemotaxis and directed swimming), to stay swimming at surfaces where receptors or favourable niches are more likely to be encountered (near surface swimming), and to sense when they have reached a desirable location and trigger changes to remain there (mechanosensing) (**Figure 2**).

2.1 Chemotaxis: the navigator directing flagellar motility

Chemotaxis is the process by which bacteria sense their environment and direct their movement (Figure 2a). This phenomenon, in which bacteria actively govern the net direction of movement so as to approach attractants and avoid repellents, was first recognized in the 1880s, and quantitative investigation began as early as the 1960s (Adler, 1966; Eisenbach, 2011). Methyl-accepting chemoreceptor proteins form large co-operative arrays that use an elegant adaptation system to increase the level of phosphorylated signalling protein CheY when traveling towards a repellent or away from an attractant (Briegel et al., 2012). In *E. coli*, phosphorylated CheY triggers a switch from a linear swim to a randomized tumble and reorientation by interacting with the flagellar C-ring to switch rotation from counterclockwise to clockwise. This behaviour results in more frequent tumbling events when proceeding in unfavorable directions. Conversely, low levels of phosphorylated CheY allow the flagellar motor to run in a counterclockwise fashion uninterrupted, lengthening runs of swimming towards favorable directions. Attractant and repellent stimuli are now known to extend beyond chemicals (chemotaxis), and include other stimuli that may be important for directed motility of pathogens, including light (phototaxis), moving fluids (rhenotaxis), osmolarity (osmotaxis), temperature (thermotaxis) and touch (thigmotaxis) (Eisenbach, 2011). The chemoreceptor system is well understood and extensively reviewed in the literature in general and for specific model organisms (Boyd and Simon, 1982; Eisenbach, 2011; Lertsethtakarn et al., 2011; Stocker and Seymour, 2012; Wadhams and Armitage, 2004).

For animal pathogens, the interaction between motility and chemotaxis directs colonization of organisms at their preferred host sites. Pathogens such as *Helicobacter pylori* and *Campylobacter jejuni* prefer to colonize mucus layers in the mammalian gastrointestinal tract. Chemotaxis allows *H. pylori* to preferentially colonize sites of gastric (stomach) injury (Aihara et

al., 2014) while chemoattractants such as mucins and glycoproteins, which are the primary constituent of mucus, lead *C. jejuni* to colonize the mucus-filled crypts in the intestine (Bolton, 2015). Other pathogens target tissue sites, with *Salmonella* spp. appearing to actively move through the mucus layer in a chemotactic manner towards the intestinal epithelium in order to inject effector proteins into host cells, making chemotaxis required for efficient colonization of the intestine in murine models (Stecher et al., 2004). Chemotaxis in *Vibrio cholera* also guides the bacteria to its preferential site of infection in the intestinal epithelium of the predominantly lower half of the small intestine, corresponding approximately to the lower jejunum and ileum. Interestingly however, in the absence of chemotaxis, *V. cholera* is capable of colonizing the entire length of the small intestine equally well and with a 10-fold decrease in infectious dose required (Boin et al., 2004; Butler and Camilli, 2005, 2004). Research into this unusual chemotaxic-deficient *V. chloera* phenotype showed that both chemotaxic and non-chemotaxic strains begin colonizing the upper half of the intestine the same way, but that chemotaxis-competent strains were attracted to the deep intervillous spaces of the intestine where they were cleared from the host by an unknown antibacterial mechanism (Freter and O'Brien, 1981). The non-chemotactic strains remained in the upper mucus gel in the upper small intestine where they likely avoid this host mechanism (Freter and O'Brien, 1981). These examples illustrate how chemotaxis can direct, and sometimes limit, the search and spreading space of a pathogen.

Chemotaxis is also relevant for plant pathogens. Chemotaxis is needed for the soil-borne plant pathogens *Agrobacterium tumefaciens* (Hawes and Smith, 1989; Merritt et al., 2007) and *Ralstonia solanacearum* (Yao and Allen, 2007, 2006) to find the correct host plant roots in their soil environments. Similarly, the plant leaf pathogen *Pseudomonas syringae* uses flagellar motility and chemotaxis for successful formation of infections on leaf surfaces (Yu et al., 2013).

Regardless if the host is plant or animal, being able to couple environmental cues to directional swimming greatly increases the likelihood of a pathogen finding its optimal infection site.

2.2 Enhancing motility by flagellar regulation as a response to the environment

While the classic chemotaxis pathway is the most commonly understood sensing/motility system, it is not the only way pathogens can sense their environments and move towards more favourable conditions. The mammalian intestinal surface is covered with a mucus glycocalyx (polysaccharide and glycoprotein covering), meaning that pathogens like enterohemorrhagic *Escherichia coli* (EHEC) must first penetrate this coating to reach and colonize the surfaces of epithelial cells. EHEC has the ability to activate motility in the large intestine upon sensing short chain fatty acids like butyrate via two of the transcriptional regulatory steps for flagellar gene synthesis (Tobe et al., 2011). This enhanced flagellum-driven motility aids EHEC in reaching the surface of the intestinal mucosa. In contrast, *V. cholera* uses a two-component sensing and response system to increase motility when bile levels are high (while the cell is in the lumen of the intestine) and decrease motility and increase virulence gene expression when bile levels are low (once the cell enters the intestinal mucus layer) (Krukonis and DiRita, 2003). These sensing and response systems provide pathogens with additional control over their motility, as they attempt to find their sites of infection.

2.3 Flagella are involved in near surface swimming

In addition to directed motility in free-swimming bacteria, flagellated bacteria can be dynamically entrapped at surfaces, continuing to swim but transiently restricted to the 2D plane described by the surface (Frymier et al., 1995; Lauga et al., 2006; Li et al., 2011; Vigeant et al.,

2002) (Figure 2b). Using three-dimensional microscopy tracking methods, cells that encountered the glass surface of a slide were often seen to spend tens of seconds exploring the surface, a behavior not seen within the bulk liquid above the surface. This phenomenon, termed near surface swimming, appears to be based on physical, hydrodynamic forces. This property has the potential of reducing a complex three dimensional search for a receptor or surface feature to a two dimensional search problem. Regardless of whether this phenomenon is a selected-for trait or an inevitable emergent property of flagellated bacteria, it is likely to play a key role in locating optimal sites of infection.

A combination of modeling and experimental work has contributed to the understanding of near surface swimming. *E. coli* cells swimming near a solid surface in viscous medium will experience two opposing hydrodynamic forces – a surface torque due to increased drag on the cell side nearest the solid surface, causing the cell to roll about its length, "pulling" the front end of the swimming cell towards the surface; and form-drag torque due to increased drag on the cell now presenting a greater cross-sectional area in the direction of flow (because of the first torque), which counteracts the rolling effect of the first torque by "pushing" the front end of the cell upwards from the surface (Vigeant et al., 2002). An equilibrium angle is achieved at the balance between these torques and this angle keeps the cell at the surface in a "nose-down" configuration. This configuration causes the cell to swim constantly "towards" the surface, leading to entrapment at the surface for periods of time. These findings were repeated and reproduced by (Berke et al., 2008), also with *E. coli*, who found an increase in cell concentration at experimental surfaces that was predicted by the model, and are also observed in other bacterial species including *Caulobacter crescentus* (Li et al., 2011) and *Vibrio alginolyticus* (Mageriyama et al., 2005).

In the context of an infection model, near surface swimming may explain aspects of *Salomonella enterica* subspecies 1 serovar Typhimurium (*S.* Typhimurium) cell invasion. During infection, a *S.* Typhimurium cell will adhere to a host intestinal cell and trigger membrane ruffling and invasion. It is known that multiple bacteria can then invade via the same ruffle but how this is achieved had remained unclear. It has now been shown that flagellar motility (but not chemotaxis) is required for reaching the host cell surface *in vitro*, and subsequent physical forces trap the pathogen for ~1.5 - 3 seconds in near surface swimming at the host cell membrane, which increases the local pathogen density and facilitates scanning of the host's surface topology (Misselwitz et al., 2012). This scanning allowed for more cells to encounter existing membrane ruffles and effectively invade the host cell via the same route. Whether this type of near surface swimming scanning is used by other flagellated bacterial pathogens remains to be studied.

2.4 Mechanosensing by flagella is used to switch developmental programs

The last major hurdle for bacterial pathogens to overcome when searching for their optimal host site is to recognize when they have arrived, to stop swimming and activate cellular pathogenesis programs such as swarmer-cell differentiation or biofilm formation. The flagellum often plays a role as a mechanical sensor relaying when a desirable surface or condition has been reached (Figure 2c). For example, flagella sense the environment to trigger changes in members of the alpha- and gamma-Proteobacteria and some Firmicutes to differentiate into swarmer-cells, a step important for pathogenesis (Kearns, 2010). In *E. coli*, dramatically increasing the load on a flagellar motor, which mimicks moving into a very viscous mucus environment, results in an increase in motor-associated stators complexes, stator remodeling and swarmer-cell differentiation, implying that the stators are the mechanosensing mechanisms (Lele et al., 2013).

Stators also appear to sense viscosity changes for the *Vibrio parahaemolyticus* motor and respond by altering flagellation patterns (Kawagishi et al., 1996). For *Proteus mirabilis*, viscosity-dependent sensing appears to use the FliL protein (found in the flagellar basal body) to activate swarmer-cell differentiation (Lee et al., 2013), while *V. cholera* can lose their flagella while passing through the mucus glycocalyx, leading to downstream virulence gene expression (Liu et al., 2008). Finally, the flagellum is a known mechanosensor for biofilm differentiation at infection sites, with pathways in *Pseudomonas aeruginosa, V. cholera, V. parahaemolyticus* and *P. mirabilis* well investigated and reviewed (Belas, 2014). Similar to sensing for swarmer-cell differentiation, sensing for biofilm formation involves the function of the flagellar motor stators. Conditions that alter stator function and ion flow across the inner membranes ultimately lead to regulatory control over the flagellar gene hierarchy and biofilm formation (Belas, 2014).

3.0 Flagella continue to play roles in pathogenesis after arriving at the site of infection

Although motility is no longer required upon reaching the site of infection, flagella play additional roles during infection (**Figure 3**). Various pathogens have evolved a range of interactions with their hosts during the establishment and progression of an infection, and flagella often play roles in these. Some organisms adhere to surfaces for replication, remaining in their planktonic forms while others differentiate into biofilms. Certain pathogens secrete effector molecules to alter the host site. Some prefer to work their way through tissue structures seeking out deeper niches to inhabit while still others chose to live inside host cells (either within vacuoles or free-living in the cytosol). As our understanding of each of these infectious lifestyles increases, we discover that the flagellum can play a role during all these colonization or invasion processes.

3.1 Flagella are directly involved in surface adhesion

Whether their ultimate goal is to enter or attach onto to a eukaryotic host cell, the first step for many pathogens is to adhere to the surface of their target (Figure 3a). The role of the flagellum in this process has been recognized as important and has recently been reviewed in the literature (Haiko and Westerlund-Wikstrom, 2013; Rossez et al., 2015). The most common structural component of the flagellum that is involved in adhesion is the filament. The flagellar filament has the potential to act as an excellent adhesion molecule, as it is surface-exposed and made up of 20,000+ identical flagellin proteins. *E. coli* strains have illustrated several cases where flagellin acts as the adherence molecule, including enteropathogenic *E. coli* (EPEC) in epithelial cell adhesion (Girón et al., 2002), enterotoxigenic *E. coli* (ETEC) with interaction between flagellin, EtpA (a exoprotein adhesin) and intestinal colonization (Roy et al., 2009) and the H7 flagella from *E. coli* O157:H7 in its interaction with bovine intestinal epithelium (Mahajan et al., 2009). In *P. aeruginosa*, both the flagellin and the flagellin cap protein (FliD) were clearly demonstrated as mucin adhesion molecules (Arora et al., 1998; Lillehoj et al., 2002). Interestingly, however, it was also found that flagellin-defective *P. aeruginosa* strains still adhered to mucin using some additional component of the flagellar motor, and mutational studies revealed that FliF (the MS-ring protein) was important (Arora et al., 1996). Given its cellular localization as a pore in the inner membrane, FliF was not expected to interact directly with mucin receptors, but rather to serve as a platform for later assembled flagellum components or to be an export pore for non-flagellar proteins that would go on to interact with mucin (Arora et al., 1996). In more general studies looking at the flagellum as a whole structure, it has been reported as the adhesion structure to intestinal cells for both *C. jejuni* and *Aeromonas caviae* (Kirov et al.,

2004; McSweegan and Walker, 1986). The literature contains many other studies documenting flagella, or parts thereof, as the adhesion structure between organisms and their hosts and highlights that this multifunctional machine can be as good an anchor as it is a propeller.

3.2 Flagella are key to biofilm formation and structure

One of the most protected and long-lasting forms a pathogenic bacteria can take once it has reached its infection site is to establish itself as a biofilm (Figure 3a). Biofilms are multicellular aggregates of bacteria bound by a matrix of extracellular polymers that include polysaccharide, protein, and DNA, which allows the cells to complex together and adhere to solid surfaces (Flemming and Wingender, 2010; Kolter and Greenberg, 2006). For pathogens like *V. cholera*, biofilms are highly relevant to epidemic outbreaks. *V. cholera* can form biofilms on the chitin surfaces of shellfish to a density of 10^4 cells/host, which exceeds the 10^3 cells/infectious dose required for infection (Pruzzo et al., 2008). As well, colonizing shellfish with biofilms also creates a reservoir for the bacteria between epidemics (Alam et al., 2007).

When a cell is considering the transition to a biofilm lifestyle, one of the first steps is to slow down or stop its flagella rotation. In *B. subtilis*, the EpsE protein interferes with the FliG (C-ring) - MotA (Stator) interaction as a "clutch" to disengage the motor (Blair et al., 2008). In several known Gram-negative systems, cyclic di-GMP acts as a messenger to control motor rotation. For *E. coli* and *Salmonella*, cyclic di-GMP complexes with the "braking" protein YcgR, where together they directly interfere with the FliG-MotA interaction (Boehm et al., 2010; Paul et al., 2010). *V. cholerae* and *P. aeruginosa* also involve cyclic di-GMP in flagellar motor regulation during biofilm formation and all four systems have been reviewed recently (Guttenplan and Kearns, 2013).

The transition from a free-swimming, planktonic cell to a biofilm requires flagellar motility in many systems. Flagellum-mediated motility is critical for wild-type levels of *Listeria monocytogenes* biofilm development, with both flagellum-minus and paralyzed-flagellum mutants having comparable defects in initial surface attachment and subsequent biofilm formation relative to wild type (Lemon et al., 2007). Interestingly, centrifuging both types of non-motile mutants onto a solid surface restored wild-type levels of attachment but not biofilm formation, indicating that if there was any role for *L. monocytogenes* flagella as a surface adhesin for biofilm formation, it is either minimal or dependent upon motility (Lemon et al., 2007). Flagellar motility is also important for the opportunistic, food-borne pathogen *A. caviae*, which generates both a single polar flagellum and multiple lateral flagella. Motility mutants in either flagellar system showed decreased abilities to form biofilms (by >30% of the wild-type levels) (Kirov et al., 2004). Structurally, flagella have been shown to make up one of the many components in the physical meshwork that comprises a biofilm. In *E. coli*, for example, flagella form a scaffold in the lower, post-exponential phase zone of the biofilm (Serra and Hengge, 2014) (whereas in the upper areas, flagella are replaced with amyloid curli fibrils that confer different mechanical properties on the biofilm). The study of flagellar involvement in biofilm formation is an active research area and several model systems, including *Bacillus subtilis, E. coli, P. aeruginosa, V. cholerae* and *V. parahaemolyticus* are being studied and have been recently reviewed (Guttenplan and Kearns, 2013).

3.3 The flagellar T3SS acts as a proto-injectisome

Pathogenic bacteria frequently secrete effector molecules as virulence factors to modulate host processes. The integral flagellar T3SS often acts as the secretion system for these effectors,

obviating the requirement for a dedicated injectisome T3SS (Duan et al., 2013) (Figure 3b). Phylogenetic analyses of the flagellar T3SS (the export apparatus in the basal body structure of the flagellum) and the non-flagellar T3SS (often referred to as an injectisome, or simply a "type III secretion system") have shown that both structures have a conserved core, with the most likely evolutionary scenario being the bacterial injectisome evolving from an ancestral bacterial flagellum (Abby and Rocha, 2012). While the more recent, specialized injectisome system is an important secretion apparatus in many bacteria, many pathogens still use the flagellar T3SS to directly secrete non-flagellar, virulence-associated effector proteins into their host cell environment.

Examples of effectors exported by the flagellar T3SS include YplA, a known phospholipase virulence factor from *Yesinia entericola*, which is dependent on functional flagellar T3SS, flagellar basal body and hook structures (Young et al., 1999). *Bacillus thuringiensis* uses the flagellar T3SS to secrete two of its known virulence factors, hemolysin BL and phosphatidylcholine-preferring phospholipase C (Ghelardi et al., 2002). *C. jejuni* has two classes of virulence proteins that both use flagellar T3SS for export; the *Campylobacter* invasion antigen (Cia) proteins and the FspA class of secreted proteins (Christensen et al., 2009; Konkel et al., 2004, 1999; Neal-McKinney et al., 2010). Cia proteins (including CiaB, CiaC, and CiaD) all appear to be involved in promoting internalization of *C. jejuni* for host invasion and require a full-length flagellar filament for proper secretion (Konkel et al., 2004; Neal-McKinney and Konkel, 2012; Samuelson et al., 2013; Ziprin et al., 2001) while FspA proteins appear to only require the flagellar T3SS, basal body and hook structures of the flagellum for secretion, and at least one variant, FspA2, has been shown to rapidly induce apoptosis of cells in cell culture *in vitro* (Poly et al., 2007). These findings indicate that besides being the apparatus that assembles

the flagellum structure, the flagellar T3SS is also a general export system for secretion of proteins that influence bacterial-host interactions.

3.4 Rotating flagella drive bacterial penetration between cell-cell junctions

Some bacterial pathogens are not content to establish infection at the surface of a host tissue but chose to penetrate into deeper tissue structures. One way this can be achieved is by boring between cell-cell tight junctions. *Helicobacter felis* exhibits the characteristically strong motility of the epsilon-proteobacteria, which has been suggested to enable it to push into tissues (Lee et al., 1988). Additionally, pathogens that fall into the bacterial order Spirochetes (like *Borrelia burgdorferi*, the agent of Lyme disease and *Treponema pallidum*, the agent of syphilis) are particularly prominent examples for exploiting their unique periplasmic endoflagellar motility for the process of penetrating endothelial monolayers (Comstock and Thomas, 1991; Thomas et al., 1988). These organisms have a dedicated review in this special issue and the interested reader is directed there for a full discussion.

3.5 Flagella do not mechanically bore through cell membranes

Although it might be imagined that forceful swimming motility could lead to host cell invasion by directly pushing the pathogen through the cell membrane, this is not the case. Plasma membranes are, in fact, a tough barrier to micron-sized objects. Work with particle bombardment of micron-sized gold spheres into eukaryotic cells (termed biolistics) reveals that velocities in excess of 100 m/s are necessary to penetrate cells, orders of magnitude greater than the ~10-100 µm/s (or 0.00001-0.0001 m/s) swimming speeds of bacterial cells (Huang and Chen, 2011; Kikkert et al., 2005; Rinberg et al., 2005; Zhang et al., 2014). In terms of force, direct

391 measurement of a swimming *E. coli* cell has revealed that it can generate a thrust force of around
392 0.57 pN (Chattopadhyay et al., 2006), whereas a force of 1.5 nN (more than 2000-fold greater)
393 was only able to dent a fibroblast membrane 500 nm inwards (not puncture it) using atomic force
394 microscopy (Thomas et al., 2013). Together these measurements orient our understanding and
395 demonstrate that flagella are incapable of ever exerting the brute force necessary to invade a cell,
396 and thus more subtle 'molecular subterfuge' strategies are required.
397

398 **3.6 Phagocytosis/Invasion**

399 Bacterial pathogens that invade host cells for replication do so by complex mechanisms
400 that actively induce their own uptake by phagocytosis into normally non-phagocytic cells (such
401 as intestinal epithelial cells) and either remain in a vacuole (e.g., *Salmonella*) or escape into the
402 cytosol for replication (e.g., *Listeria* and *Shigella*) (Cossart and Sansonetti, 2004) (Figure 3c).
403 These invasive strategies allow pathogens to avoid many host immune defenses and establish
404 productive infection having evolved to survive and thrive inside the host cell. Phagocytosis for
405 entry into the host cell is carried out by either the zipper or trigger mechanism, both of which are
406 well understood and have been reviewed (Cossart and Sansonetti, 2004; Sansonetti, 2001).
407 Similar to many other stages of infection, flagellar motility has been shown to be necessary for
408 proper invasion of many pathogens through phagocytosis.

409 There are several examples where non-motile flagellar mutants have severely reduced
410 invasion ability. *Burkholderia cepacia*, *C. jejuni* and *P. mirabilis* are all invasion-compromised
411 when flagellar motility is abolished (Grant et al., 1993; Mobley et al., 1996; Tomich et al., 2002).
412 However, when *B. cepacia* or *P. mirabilis* are centrifuged onto their host cells (without active
413 motility), *P. mirabilis* was then able to invade its host cell while *B. cepacia* still could not

(Mobley et al., 1996; Tomich et al., 2002). This indicates that *B. cepacia*'s invasion is dependent on an active motility process independent of chemotaxis and flagellar adhesion to the host.

Legionella pneumophila is an interesting pathogen that usually inhabits freshwater biotopes by living as an intracellular pathogen of amoebae. However, if aerosolized and inhaled, it can invade and multiply in alveolar macrophages and non-phagocytic cells in humans to cause Legionnaries' disease. *L. pneumophila* flagellar mutants have been made and they were determined to have no effect on cell adhesion or intracellular rate of replication (Dietrich et al., 2001; Molofsky et al., 2005). However, loss of flagellar motility moderately reduced invasion efficiency in amoebae and severely reduced the invasion efficiency in a human macrophage-like cell line (Dietrich et al., 2001). So, while flagellar motility is necessary for efficient invasion of *L. pneumophila* into all its hosts, flagellar loss had a greater impact on its internalization with its mammalian host cell type.

From a host immune response perspective, professional phagocytosis cells actively try to seek out and engulf pathogens. An interesting set of studies in *P. aeuroginosa* revealed that innate immune cells respond to motility, not just the flagellar structure, as targets for phagocytosis (Lovewell et al., 2014, 2011). This was determined by generating stator mutants in *P. aeuroginosa* strains, so flagellar structures were present but motility was abolished; non-motile strains with paralyzed flagella were ~100-fold more resistant to phagocytosis than motile, wild-type strains (Lovewell et al., 2011). This phagocytosis resistance was not due to a measurable change in the expression of common outer membrane proteins or known regulators of pathogen-associated molecular patterns (PAMPs), but rather that phagocytic cells responded to bacterial swimming as a function of flagellar rotation after initial contact and that phagocytosis is directly proportional to the flagellar torque of the bacteria.

To address how actual motility, and not just the presence of the flagella, might affect phagocytosis, two reasonable theories have been proposed; either bacterial motility alters the expression of unknown bacterially-produced factors or ligands that alters phagocyte recognition or that cells can "sense" motility and respond via phagocytosis (Lovewell 2011). Investigation of *P. aeuroginosa* indicated that there was no significant change in gene expression that correlated with loss of motility and phagocytic susceptibility, leaving an obvious motility/phagocytic factor as yet undiscovered (Lovewell 2011). Alternatively, innate immune cells may be able sense bacterial motility through membrane depression or activation of an unknown tension receptor(s), and that this mechanical perturbation could activate phagocytosis (Lovewell 2011). There are examples of cellular mechanosensory systems in other physiological systems, such as cellular stretch detection in muscle sarcoma cells (Birukov et al., 1995) and shear-enhanced adhesive catch bonds in rolling leukocytes (Finger et al., 1996), but to date no reports have identified such a mechanism contributing to pathogen recognition.

4.0 For growth and maintenance with the host, pathogens must have a strategy to deal with the immunogenicity of their flagella.

Once a pathogen has reached its desired site of infection and has established itself either on or inside its host, the next challenge faced is avoiding the host immune defense system long enough to grow and replicate. Conserved from worms to mammals, the eukaryotic innate immune system includes sets of germline-encoded pattern-recognition receptors (PRRs) to automatically recognize and respond to microorganisms. These PRRs recognize microbial components, known as PAMPs, which are highly conserved bacterial components/structures. The bacterial flagellin protein has a highly conserved 13 amino acid core structure required for

460 protofilament formation and assembly, which makes it an ideal PAMP (Smith et al., 2003). For
461 sensing PAMPs outside the mammalian cell, the immune system uses Toll-like receptors (TLRs)
462 (Akira et al., 2006). TLR-5 is dedicated to the recognition of extracellular bacterial flagellin
463 protein (Hayashi et al., 2001). If flagellin protein is detected within the cytosol of a cell, it is
464 detected through a different innate immune pathway; the Nod-like receptor (NLR) Ipaf, which
465 activates caspase-1 and interleukin 1β, or Naip5 (Miao et al., 2007, 2006, Ren et al., 2006). This
466 means that for pathogenic bacteria to survive the eukaryotic host's innate immune system and
467 thrive, they must either reduce or turn off their flagellar expression or evade the immune system
468 by hiding their flagella from it (**Figure 4**). Depending on how essential flagellar motility is for
469 the pathogen at the replicative stage of infection, different organisms take different approaches.
470

471 **4.1 Some pathogens reduce or eliminate flagellar expression**

472 The obvious solution to flagellin-mediated immune clearance of bacteria is for the
473 bacterium to simply turn off flagellar expression when it no longer needs it, a response that is
474 common and widespread (Figure 4a). The normal microbiota within the mammalian gut has been
475 shown to have overall low levels of flagellin expression, while $TLR5^{-/-}$ mice showed a diversity
476 of gut microbiome members with overexpressed flagellar genes (Cullender et al., 2013).
477 Commensal strains of motile *E. coli* introduced into the mouse gut were found to lose 45-50% of
478 their motility by day three after feeding and between 80-90% of their motility by day 15 (Gauger
479 et al., 2007). The same pattern is seen with pathogenic strains, with *S.* Typhimurium strongly
480 down regulating its genes coding for flagellar machinery and chemotaxis when intracellular in
481 macrophages during infection (Eriksson et al., 2003). This response is similar for plant pathogens
482 as well, where the gene expression profiles of *P. syringae* show that they give up their motility in

favor of replication processes once they have established themselves inside the leaf cell (Yu et al., 2013).

One interesting mechanism to control this downregulation of flagellar motility genes once inside the host is temperature sensing. Both *L. monocytogenes* and *L. pneumophila* demonstrate temperature-dependent expression of their flagella (Kamp and Higgins, 2011; Ott et al., 1991). Under environmental temperature conditions (22°C to 30°C), both systems express flagella, but when raised to 37°C, flagellar expression is markedly reduced. In the *L. monocytogenes* system, it was determined that the protein GmaR acts as a protein thermometer that controls temperature-dependent transcription of flagellar motility genes (Kamp and Higgins, 2011). These types of systems provide a pathogen with the ability to turn off immune-stimulating antigens before they can trigger adverse host defenses for the pathogen once inside their target host.

4.2 Some pathogens utilize immune evasion strategies

Organisms that continue to express their flagella during their time inside a host have developed many ways to avoid the immune system, either by alternating their expressed flagellin proteins regularly (phase variation), having different subsets of the population express flagella and not (bistability), by altering the flagellin protein structure to be unrecognizable to TLR5 (flagellin modification) or adding post-translational modifications to flagellins to mask target sites (glycosylation).

S. Typhimurium alternately expresses two different flagellar filament proteins, FljB and FliC, in a process known as flagellar phase variation (Andrewes, 1922; Bonifield and Hughes, 2003). The molecular mechanism mediating flagellar phase variation occurs by a site-specific

DNA inversion event in the chromosome, allowing alternative expression between the flagellins at a rate of 10^{-3} to 10^{-5} per cell generation (Stocker, 1949). While altering flagellin expression in this way does not change the innate immune system's ability to recognize the flagellum, the different flagellin subunits do have different antigenicities, making them harder for the cellular immune response to clear out effectively (Bonifield and Hughes, 2003).

Another mechanism that utilizes flagellar gene expression is bistability, where a clonal group of cells demonstrate two distinct motility phenotypes within the population; motile and non-motile. For *S.* Typhimurium cells, bistability is observed when the cells are in the environment, where nutrient levels control the proportion of motile/non-motile cells (Koirala et al., 2014), and during infection, where different proportions of inflammatory (motile) and non-inflammatory (non-motile) cells influence systemic spread (Steward and Cookson, 2012). *Bacillus subtilis* is another well-studied example, with the population differentiating into either non-motile chains that form biofilms and resist protozoan grazing or motile cells that disperse to new, potentially more favorable niches (Mukherjee and Kearns, 2014). In most cases, bistability is seen as a bet-hedging strategy to optimize the population's chance of survival (Steward and Cookson, 2012).

Another immune avoidance mechanism for bacteria is to alter their flagellin sequence to be unrecognizable by TLR5 (Figure 4b). The TLR5 recognition site was determined to be within amino acids 89-96 of the N-terminal D1 domain of the flagellin protein (Andersen-Nissen et al., 2005). It was found that flagellin from *C. jejuni, H. pylori* and *Bartonella bacilliformis* have alterations to these amino acids that abolishes TLR5 recognition, as well as complementary mutations elsewhere in the flagellin protein to maintain filament formation and motility (Andersen-Nissen et al., 2005; Watson and Galán, 2005). When these mutations were transferred

529 into a *S.* Typhimurium flagellin sequence, which is normally strongly recognized by TLR5, the
530 flagellin evaded TLR5 recognition and the bacteria remained motile (Andersen-Nissen et al.,
531 2005).

532 Finally, another way to modify flagellins to evade the immune system is through post-
533 translational modification (Figure 4c). Glycosylation, the addition of carbohydrate moieties to
534 the protein backbone, is a common bacterial surface protein modification and the flagellins of
535 many bacteria, including *C. jejuni*, *P. aeruginosa*, *Burkholderia cenocepacia* and *Aeromonas*
536 *hydrophila* are known to be glycosylated (Brimer and Montie, 1998; Ewing et al., 2009;
537 Hanuszkiewicz et al., 2014; Merino et al., 2014; Thibault et al., 2001). *C. jejuni* flagellins are
538 modified at 19 different sites on its major flagellin protein and it has been speculated that the
539 structural similarity of the flagellin glycans (which include 5-acetamidino-7-acetamido-
540 pseudaminic acid) to the predominant sialic acid found in mammalian cells (which is N-
541 acetylneuraminic acid) may play a role in immune avoidance (Thibault et al., 2001). For *B.*
542 *cenocepacia*, its flagellar glycosylation clearly led to a reduced inflammatory response in the
543 host by reducing TLR-5 recognition (Hanuszkiewicz et al., 2014). Overall, the effects of
544 glycosylation on the immune recognition of flagellins is still an active area of research and
545 remains to be better understood in the future.
546

547 **5.0 Dispersal**

548 After a successful growth phase inside its host, the final step a pathogenic bacterium
549 needs to accomplish is to disperse to find new hosts to colonize. This dispersal is commonly
550 motility-mediated, which often requires the reactivation of flagellar systems after their down-
551 regulation during the growth and maintenance phase of infection.

552

553 **5.1 Reinitiate flagellar expression for escape**

554 Motility is often necessary for pathogen escape from intracellular host cells back into the
555 general host environment. *S.* Typhimurium reactivates its motility while still intracellular to
556 prepare for exit from infected macrophages. In conjunction with inducing eukaryotic cell death,
557 intracellular *Salmonella* bacilli intermittently exit host cells in a flagellum-dependent manner,
558 exemplified by the observation that highly motile *S.* Typhimurium could escape from host cells
559 while non-motile Δ*fliA* mutants could not (Sano et al., 2007). Uropathogenic *E. coli* (UPEC)
560 cells establish their infection inside the superficial umbrella cells in the bladder, where they form
561 complex intracellular bacterial communities (IBCs), similar to biofilms. During the growth
562 phase, bacteria in IBCs are non-motile and develop into highly organized biofilm-like
563 communities that ultimately fill most of the host cytoplasm. When the IBC is mature, the host
564 cell undergoes apoptosis and the bacteria switch back to a motile phenotype allowing detachment
565 from the IBC and eventual fluxing out of the host cell through areas of compromised cell
566 membrane integrity (Justice et al., 2004). The motility of intracellular and fluxed UPEC cells
567 was characteristic of flagellar-based motility based on video microscopy (Justice et al., 2004).

568 In addition to using flagellar motility to escape intercellular host spaces to exit into the
569 exterior environment, some pathogens use their motility to move between host cells to spread
570 during infection within a single host organism. Several *Burkholderia* species invade mammalian
571 cells via phagocytosis, escaping their endosomes and replicating in the cytoplasm accompanied
572 by actin-based motility and cell–cell spreading, analogous to *Shigella flexneri* and *L.*
573 *monocytogenes* infections (French et al., 2011). Mutational analysis in *Burkholderia*
574 demonstrated that MotA2 (stator)-dependent flagellar motility could drive intercellular spread

independently of BimA-mediated actin polymerization, and that flagellar-mediated motility increased the frequency of contact between bacteria and host cell membranes; such contact was a prerequisite for membrane fusion and cell-to-cell spreading of *Burkholderia* within its host (French et al., 2011).

Flagellar motility is not only required by animal pathogens for escape, but also for bacterial pathogens of other bacteria. The bacterial intracellular pathogen *B. bacteriovorus* is a Gram-negative bacterium that preys on other Gram-negative bacteria by invading prey cells and replicating in their periplasmic space. Flagellar motility is required for the extracellular attack phase and the escape phase of their life cycle, while growth phase cells are non-motile and non-flagellated. Using anti-sense RNAs to degrade stator protein transcripts, it was shown that *B. bacteriovorus* cells that were unable to reinitiate flagellar expression after their growth phase were compromised in host cell exit (Flannagan et al., 2004).

5.2 Inducing an immune response and host cell death as an escape and reinfection mechanism

An interesting example where a pathogen actively induces an immune response as part of its dispersal and spreading strategy is with *Salmonella*. During *S.* Typhimurium infection, cells live within special vacuoles inside epithelial or macrophage cells and during their growth, they translocate flagellin proteins into the cytosol via their injectisome T3SS (Sun et al., 2007). *S.* Typhimurium uses the secreted flagellin to activate Ipaf and caspase-1, initiating host cell death via a controlled pyroptosis (Fink and Cookson, 2007; Stewart et al., 2011). Unlike apoptosis, pyroptosis produces inflammatory responses during the host cell death which recruits additional macrophages to the site of infection. These macrophages phagocytose the released *S.*

598 Typhimurium and continue the spread of infection (Fink and Cookson, 2007). In this way, the
599 flagellin proteins detected by the host cell act as a catalyst to recruit new cells to the site of
600 dispersal to be newly infected.
601
602 **6.0 Concluding remarks**
603 Flagella have evolved to play roles at all stages during pathogenesis. Flagellar motility is
604 an important process in many stages of a pathogen's life cycle. In many cases, the initial function
605 of the flagellum is to find the proper host site to initiate an infection and then leave the host site
606 to spread the infection to other cells, body sites or other hosts. When this propulsion is coupled
607 to chemotaxis, motility can be a very effective virulence factor to allow efficient colonization
608 and spread. Beyond movement, however, the flagellar motility system has become necessary for
609 some bacteria to sense their environmental conditions, adhere to target sites, invade host cells,
610 secrete effector molecules and evade the host immune system. These complex interactions
611 between bacterial flagella and the host environment have evolved over hundreds of thousands of
612 years to add utility to an existing bacterial structure. As our understanding of pathogenic life
613 cycles and processes continues to grow, it is likely that new roles for the bacterial flagellum
614 during pathogenesis will be revealed. While motility was likely a predisposing factor during
615 initial evolution of pathogenesis, it appears that flagella have become incorporated into every
616 other facet of the infection process since then.
617
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Figure 1: Schematic of the structure of the bacterial flagellum. The figure is based upon the model Gram-negative bacteria *Escherichia coli* and *Salmonella enterica*. During assembly, basal body T3SS components unfold and export subunits of the rod, hook and filament for incorporation at the cell-distal tip of the growing structure. Energy harvesting stator complexes in the basal body interact with the torque-generating C-ring to bring about rotation of the extracellular filament for motility. OM = outer membrane, PG = peptidoglycan layer and IN = inner membrane.

Figure 2: The role of flagella in reaching the host/target site. Bacteria have evolved systems in conjunction with their flagella to sense their environment and move in favourable directions. (a) Chemotaxis and directed swimming: pathogens use chemical gradients to navigate towards optimal host sites for colonisation. One example is the gastric pathogen *H. pylori*, which uses chemical gradients to selectively infect sites of existing tissue damage in the stomach. (b) Near surface swimming: upon encountering surfaces, bacteria prolong swimming interactions at the surface to facilitate target site selection. For instance, the intestinal pathogen *Salmonella* swims along the surface of cells, where receptors or favourable niches for host entry are likely to be encountered. (c) Mechanosensing: bacteria can sense when they have reached a desirable location and trigger changes that help them remain there. The bacterium *E. coli* senses increased viscosity (e.g. from protective mucus linings of hospitable tissues) to recruit additional stator complexes to its flagellar motor and express more flagella for swarming towards host cells.

Figure 3: Roles of flagella in colonizing or invading. Different pathogens have a range of interaction types with their hosts during the establishment and progression of an infection. (a) Some organisms adhere to surfaces for replication, remaining in their planktonic forms while others differentiate into biofilms. (b) Others secrete effector molecules to alter the host site. (c) Some prefer to work their way through tissue structures seeking out deeper niches to inhabit while still others chose to live inside host cells via phagocytosis (either within vacuoles or free-living in the cytosol).

Figure 4: Changes in flagella to allow for growth and maintenance during infection. For pathogenic bacteria to survive the eukaryotic host's innate immune system and thrive, there are a number of strategies employed. Some organisms, like *Salmonella* (A) alter their expressed flagellin proteins regularly (phase variation) and then turn off their flagellar expression once they have reaching the target site. Other organisms, like *Helicobacter* (B) or *Campylobacter* (C) alter their flagellin protein structure to be unrecognizable to TLR5 (flagellin modification) or add post-translational modification to flagellins to mask target sites (glycosylation), respectively. The goal of these modifications is to modulate or avoid the host immune system to allow for a productive infection.