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Investigation of Bacteroidetes Gliding Motility

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0.1 Abstract

Bacteroidetes gliding motility is a type of surface motility in which rod-shaped bacteria move up to $2 \frac{\mu m}{s}$ in a corkscrewing motion. *Flavobacterium johnsoniae* is the primary model organism for the study of *Bacteroidetes* gliding. SprB is the main adhesin in this organism and moves in a helix along the cell surface. This movement is guided by an underlying track that is anchored to the inner leaflet of the outer membrane. The essential gliding lipoprotein GldJ, which is helically arranged when visualised in fixed cells, is suggested to form this track. However, direct *in vivo* imaging of GldJ is yet to be achieved. Two currently outstanding questions about *Bacteroidetes* gliding motility are 1) how adhesion of SprB to the substratum is controlled so that binding only occurs when moving from the leading to the lagging cell pole and 2) how/if the cell discriminate between the poles.

In this thesis, a fusion of the HaloTag domain to SprB enabled labelling of SprB with stable and bright dyes. The movement of SprB could then be visualised using single-particle tracking to reveal the underlying track topology. These tracking data suggest that the underlying track is not a single closed loop currently proposed, but rather a complex and potentially dynamic structure that can form multiple loops and cover most of the cell surface.

SprB is encoded by the *sprB* operon that further encodes RemFG, Fjoh_0982, and SprCDF. In this thesis I show that all these components, except *fjoh_0982*, are required for gliding motility but only *sprF* are required for SprB helical movement. All the *sprB* operon components required for gliding are also required for SprB-mediated attachment to glass, indicating that they regulate adhesion of SprB. RemG and SprCD move in a helix reminiscent of the SprB movement pattern. The helical movement does not depend on SprF or SprB, but rather on the SprF-homologous N-terminal domain of SprD. Observations of gliding cells with fluorescently labelled SprC revealed accumulation of SprC near the leading cell pole. This polar accumulation correlated with the direction of movement and was not observed in cells that did not move. Furthermore, a mutant lacking the C-terminal 50 residues of SprD was unable to accumulate SprC at the leading pole. SprB did not show a similar asymmetric distribution in gliding cells.

Fluorescence microscopy shows that helically moving *sprB* operon proteins accumulate at midcell in dividing cells in a GldJ dependent manner. Cross-linking mass spectrometry indicates that GldJ interacts with the *sprB* operon proteins as well as GldKNO, essential outer membrane components of the type 9 secretion system which is a pre-requisite for *Bacteroidetes* gliding motility.

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0.3 List of abbreviations

Abbreviation	Explanation
aa	amino acids
A-motility	Adventurous-motility
ABC	ATP-binding cassette
ACN	ACetoNitrile
ATP	Adenosine TriPhosphate
CM	Cytoplasmic Membrane
CCCP	Carbonyl Cyanide m-ChloroPhenylhydrazone
CTD	C-Terminal Domain
CXMS	Cross-Linking Mass Spectrometry
CYE	Casitone Yeast Extract
CW	Cell Wall
DDM	n-Dodecyl- β -D-Maltopyranoside
DMSO	DiMethyl SulfOxide
DNA	Deoxy Ribonucleic Acid
DSS	DiSuccinimidyl Suberate
DTT	DiThioErythritol
EDTA	EthyleneDiamineTetraacetic Acid

Abbreviation	Explanation
ESI-MS	ElectroSpray Ionisation Mass Spectrometry
FAC	Focal Adhesion Complex
GAM	Generalised Additive model
GDP	Guanosine DiPhosphate
GLS	Generalised Least Squares
GTP	Guanosine TriPhosphate
HILO	Highly Inclined and Laminated Optical sheet
HMM	Hidden Markov Model
KLD	Kinase Ligase DpnI
LB	Luria-Bertani
LDS	Lithium Dodecyl Sulfate
LPS	LipoPolySaccharide
MCP	Methyl-accepting Chemotaxis Protein
ML	Machine Learning
MM	Motility Medium
MSD	Mean Squared Displacement
NTA	NitriloTriacetic Acid
OM	Outer Membrane

Abbreviation	Explanation
ON	Over Night
PAGE	Poly-Acrylamide Gel Electrophoresis
PMF	Proton Motive Force
PCR	Polymerase Chain Reaction
PPI	predicted Peptidyl-Prolyl cis/trans-Isomerase
PSF	Point Spread Function
PY2	Peptone Yeast 2
RNA	RiboNucleic Acid
RT	Room Temperature
S-motility	Social-motility
SDS	Sodium dodecyl sulphate
SHILO	Strobed Highly Inclined and Laminated Optical sheet
SPT	single-particle Tracking
sfGFP	superfolded green fluorescent protein
YFP	Yellow Fluorescent Protein
T4P	Type 4 Pili
T9SS	Type 9 Secretion System
TEAB	Tetraethylammonium bromide

Abbreviation	Explanation
TFA	TriFluoracetic Acid
TPR	TetratricoPeptide Repeat
WT	Wild Type

Chapter 1

Introduction

Organisms exist in constantly changing environments, and the ability to move between them can be key for survival. Bacteria without the ability to move actively are dispersed by Brownian motion, a type of random movement resulting from constant collisions by the surrounding molecules. The time it takes to travel increases as a square of the distance. For example, a molecule moves across a $1\text{-}\mu\text{m}$ bacterial cell in 0.5 ms but across 1 mm in 1000 s (Mitchell, Kogure, 2006). Directed bacterial movement is normally achieved using dedicated molecular machineries. However, it is also possible for bacteria to move using a more passive approach. An example of this is sliding where cells move using the force of cell growth (Hölscher, Kovács, 2017). The various types of bacterial motility and their underlying systems

are listed in table 1.1.

Table 1.1: Currently identified bacterial motility systems. PMF, proton motive force. T4P, Type IV Pili.

Name	Speed [$\mu\text{m/s}$]	Energy	References
Motility in liquid			
Swimming via flagella	<i>Escherichia coli</i> 14-35	PMF	(Nakamura,
	<i>Thiovulum majus</i> 600		Minamino,
			2019)(Berg, Brown,
			1972) (Lowe et al.,
			1987)((Garcia-Pichel,
			1989))
cyanobacterium	<i>Synechococcus</i>	PMF	(Waterbury et al.,
swimming	sp. 5-25		1985)
Spiroplasma	<i>Spiroplasma mel-</i>	ATP(?)	(Shaevitz et al.,
swimming	<i>liferum</i> 3.3-4.7		2005)(Sasajima,
			Miyata, 2021)
Motility in semi-solid medium			
Swarming via flagella	<i>Escherichia</i>	PMF	(Nakamura,
	<i>coli</i> 2-10(50)		Minamino, 2019)
Motility on solid medium			
Twitching via T4P	<i>Pseudomonas</i>	ATP	(Craig et al.,
	<i>aeruginosa</i> 0.5		2019)(Skerker, Berg,
			2001)

Name	Speed [$\mu\text{m/s}$]	Energy	References
Gliding via Crawling	<i>Mycoplasma mobile</i> 1-4.6(7.7)	ATP	(Jaffe et al., 2004)(Rosengarten, Kirchhoff, 1987)
Gliding via protein adhesins	<i>Myxococcus xanthus</i> 0.067	PMF	(Nan, Zusman, 2016)(Wu et al., 2009)
(<i>Myxobacteria</i>)			
Gliding via protein adhesins	<i>Flavobacterium johnsoniae</i> 1-3	PMF	(Nan, Zusman, 2016)(Nakane et al., 2013)
(<i>Bacteroidetes</i>)			
Gliding via an unknown mechanism	<i>Chloroflexus aggregans</i> 1-3	?	(Hanada et al., 1995)

Movement in liquids (swimming) and semi-solid agar (swarming) is most often achieved using well-studied flagella systems. The flagellum is a rotating protein polymer that propels the bacterium forward (Trachtenberg et al., 2003). There are many subtypes of flagellum-mediated motility systems. However, all of them depend on the rotation of one or more flagella driven by the ion-motive force across the cytoplasmic membrane (CM). In contrast to flagellum-mediated swimming, the less well understood swimming motility of the cyanobacterium *Synechococcus* sp. does not depend on extracellular appendages but is instead proposed to use helically

travelling surface waves of surface-layer proteins (Ehlers, Oster, 2012)(Waterbury et al., 1985).

Bacterial movement on solid surfaces can be achieved independent of flagella with one of a number of other distinct mechanisms. Twitching motility is the best-studied and most widely conserved type of surface motility. It consists of consecutive rounds of extension, attachment, and retraction, of adhesive type 4 pili (T4P). The remaining types of movement on solid surfaces are all classed as "gliding motility". Historically, gliding motility has been defined and referred to in multiple ways and early works have also been referred to as creeping or crawling. Pringsheim (1951) defined gliding as "a translocation along solid bodies or the interface between water and air, during which no wriggling, contraction or peristaltic alterations are visible, the change of shape being restricted to bending". Later definitions put more emphasis on the lack of obvious motility appendages such as pili or flagella (Henrichsen, 1972). It is now clear that there are several distinct types of gliding motility. Gliding via crawling refers to the centipede-like movement of light-bulb shaped mycoplasma cells using a collection of tiny (50 nm) legs that catch, pull, and release the surface (Miyata, Hamaguchi, 2016). *Chloroflexus aggregans* gliding motility is mostly unstudied. However, it was recently reported that poly-lysine coated beads moved along the length of *C. aggregans* cells suggesting that adhesive complexes are moving along the cell surface (Fukushima

et al., 2016). *Myxobacteria* and *Bacteroidetes* gliding motility both utilise a helical forward-rolling movement to glide across surfaces. Despite the similarity from a macroscopic perspective, it has become increasingly clear that gliding motility of the *Myxobacteria* and *Bacteroidetes* phyla are achieved through different molecular types of machinery.

In this thesis, I am studying the *Bacteroidetes* gliding motility machine. However, with similar functional traits comes similar challenges and limitations. Thus, in order to understand the molecular mechanics of *Bacteroidetes* gliding motility, it is important to capitalise on the knowledge obtained for *Myxobacterial* gliding.

1.0.1 *Myxococcus xanthus* motility

The Deltaproteobacterium *Myxococcus xanthus* is the model organism for *Myxobacteria* gliding motility. It is a social, predatory, spore-forming species capable of fruiting body formation (Cao et al., 2015)(Muñoz-Dorado et al., 2016). This complex uni- and multi-cellular lifestyle requires a sophisticated motility apparatus. *Myxococcus xanthus* cells have two types of motility systems: social (S), and adventurous (A) (Hodgkin, Kaiser, 1979). S-motility is also known as twitching motility and is mediated by T4P. The pilus can not only attach to the surface but also to neighbouring cells, which in turn allows *M. xanthus* to move in swarms (Mattick,

2002)(Wu, Kaiser, 1995)(Kaiser, 1979). A-motility, also known as gliding motility, is usually observed when the cell density is low and the cell is exploring its nearby environment. Although the two motility systems seem to be dedicated to different functions they are synergistic, as cells possessing both achieve $\sim 50\%$ greater colony spreading speed than cells lacking either A or S motility (Kaiser, 2007). In gliding motility, *M. xanthus* cells move with a constant rolling motion, occasionally flipping the direction of movement (Spormann, 1999)(Muñoz-Dorado et al., 2016)(Nan et al., 2014). Gliding motility works by assembling a focal adhesion complex (FAC) at the leading cell pole that attaches to the substratum while moving in a helix along the cell body until it reaches the lagging cell pole. The FAC disassembles and releases the substratum, thereby ensuring that force is only generated in one direction. To understand how *M. xanthus* is capable of gliding motility a review of the molecular components is required.

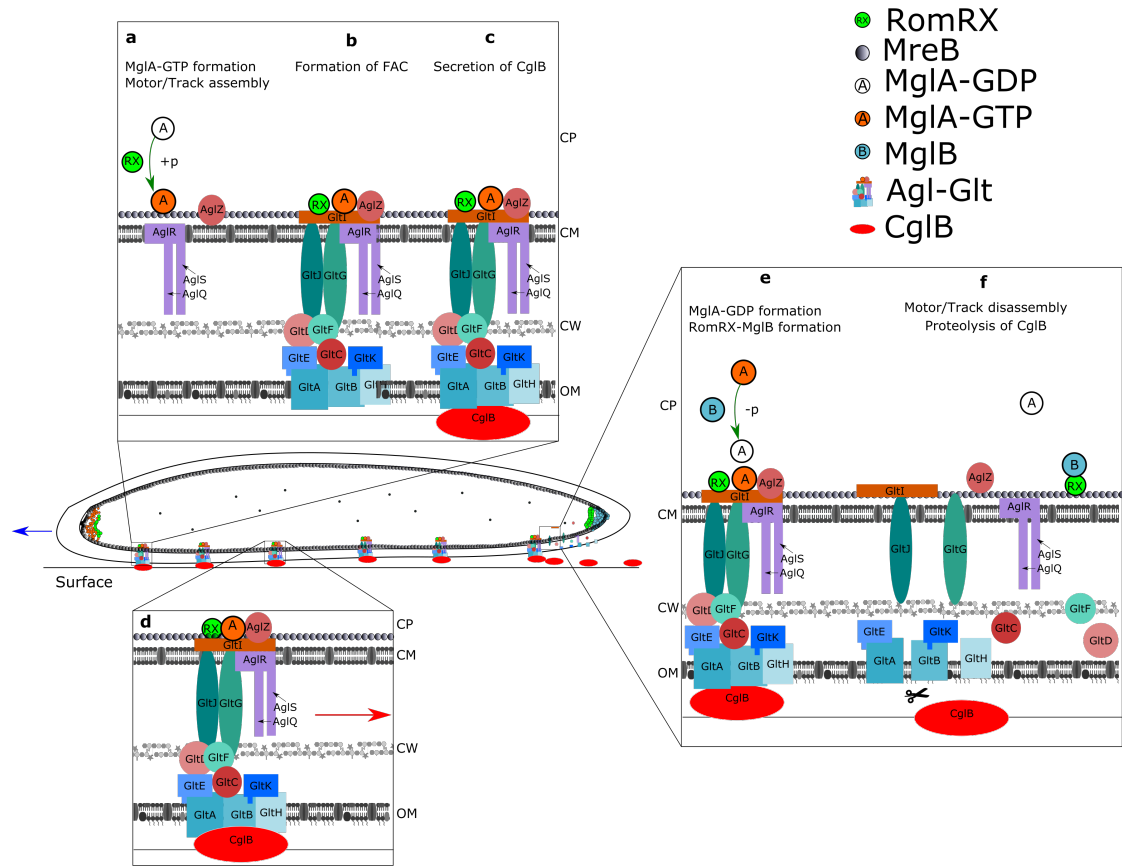


Figure 1.1: Model of *M. xanthus* gliding motility. Focal adhesion complexes (FACs) are formed at the leading pole (**a-c**), propelled along the cell surface (**d**), and disassembled at the lagging pole (**e-f**) (**a**) Diagram of FAC assembly. MgIA-GDP is phosphorylated by RomRX, resulting in MgIA-GTP. (**b**) The MgIA-GTP-RomRX complex stimulates the assembly of the FAC which (**c**) secretes the adhesin CglB. (**d**) The fully assembled FAC, attached to the surface via CglB, is propelled towards the lagging pole by the proton motive force, transduced by AglRQS. (**e**) MglB stimulates MgIA-GTP hydrolysis to form MgIA-GDP at the lagging pole. (**f**) MgIA-GDP is unable to bind MreB and the FAC components, leading to the disassembly of the FAC. MgIA-GDP diffuses through the cytoplasm, back to the leading pole. Disassembly of the FAC at the lagging pole leads to proteolysis of CglB which gets left behind and forms a trail. RomRX binds MglB and is retained at the lagging pole.

The *M. xanthus* gliding machine

The *M. xanthus* gliding machine propels focal adhesion complexes (FACs) from the leading to the lagging pole in a helix. The helical path of the gliding machine is dictated by the actin-homologous cytoskeletal protein MreB which is located on the interior face of the CM and known in many other bacteria for its role in cell-wall synthesis and cell shape (Shi et al., 2018). In *M. xanthus*, MreB moves faster (up to $2.5\ \mu\text{m/s}$) and with a different pitch angle relative to the long cell axis ($\sim 25^\circ$ vs. $\sim 90^\circ$ in *E. coli*) than observed in other rod-shaped bacteria (Fu et al., 2018), suggesting that MreB in *M. xanthus* has evolved to become part of the gliding machine.

To propel the FAC along the MreB track, the gliding machine uses the flagellar motor stator homologous proteins AglRQS. AglR is homologous to the stator protein MotA and AglQS are both paralogs of the stator protein MotB. Together these proteins transduce the energy of the proton motive force (PMF) across the CM into the mechanical energy used to drive gliding (Nan et al., 2013)(Sun et al., 2011). Unlike MotB, AglQ and AglS lack the C-terminal peptidoglycan binding domain of MotB (Nan et al., 2013) and should be free to move. MreB moves with a speed and angle that correlates with AglR and deletion of *aglQS* results in immobile MreB (Fu et al., 2018)(Nan et al., 2013). Thus, the AglRQS proteins move along and

together with the MreB track. Single-molecule labelled AglR revealed that AglR slowed down near the leading pole and this occurred more frequently as the authors increased the hardness of the surface on which the cells moved (Nan et al., 2013).

The current model for *M. xanthus* gliding suggests that the AglRQS motor proteins associate with so-called gliding transducer (Glt) components, the CglB adhesin protein, the AglZ coiled-coil cytoplasmic protein, and a few other proteins related to pole identity and directionality (discussed later in this chapter) at the leading pole, forming the FAC.

After the formation of the FAC, it moves along the MreB track towards the lagging pole where it disassembles (Faure et al., 2016). Upon FAC formation the Glt components of the FAC secrete the CglB adhesin, thus ensuring the attachment of the FAC to the substratum for the duration of FAC movement from leading to lagging pole. At the lagging pole, CglB is cleaved by metalloproteases to end FAC adhesion, in a process regulated by the OM Glt proteins of the FAC, and CglB fragments can be found in the slime-trails deposited by moving *M. xanthus* cells (Islam et al., 2020).

The Glt complex is encoded by a total of 11 genes: *gltABCDEFGHIK* (Luciano et al., 2011), distributed across two operons. Eight of these genes, *gltA-H*, are paralogs of the spore-forming genes *nfsA-H*, which the AglRQS complex also interacts

with during spore formation (Wartel et al., 2013). A schematic illustration of the gliding machine can be seen in figure 1.1.

The structure of the gliding transducer is still to be fully resolved. GltA, GltB and GltH are predicted OM β -barrel proteins and GltA and GltB additionally contains a TonB-box (Faure et al., 2016). These proteins are thought to form the OM platform that secretes CglB (Figure 1.1, **b**) (Islam et al., 2020). GltC, GltD, and GltE are periplasmic tetratricopeptide (TPR) repeat-containing proteins (Luciano et al., 2011) and GltF is a periplasmic protein with a TonB-box (Faure et al., 2016)(Luciano et al., 2011). These proteins likely act as a bridge from the OM GltABH proteins to the cytoplasmic and cytoplasmic membrane (CM) proteins (Figure 1.1, **b**). GltG and GltJ are both CM proteins with a single transmembrane helix and a periplasmic coiled-coil structure with a TonB-box (Faure et al., 2016). GltI is a cytoplasmic protein with two TPR repeats (Luciano et al., 2011). Little is known about GltI and GltJ. GltG interacts with the motor protein AglR (Wartel et al., 2013), linking the motor to the Glt complex (Figure 1.1, **b**).

Controlling the direction of movement

M. xanthus gliding is involved in advanced multicellular behaviour such as fruiting body formation. This requires the bacteria to tune and control the direction of

gliding. In order for *M. xanthus* cells to control their direction of movement, the identity of the leading and lagging poles must be known and controlled to ensure that the FACs are assembled at the leading pole and disassembled at the lagging pole, resulting in net movement. The switching of the cell pole identities further allows the cell to reverse the direction of movement, which can be further controlled by tuning the frequency of the reversals.

Cell pole polarity is achieved by the Ras-like GTPase, MglA, and its cognate GTPase-activating protein, MglB, as well as the guanine nucleotide exchange factor, RomX in combination with the response regulator RomR (Schumacher, Sogaard-Andersen, 2017)(Szadkowski et al., 2019). RomR localises to both poles and is capable of forming complexes with either of MglA and MglB *in vitro* (Figure 1.1) (Keilberg et al., 2012).

RomRX-MglA-GTP helps *M. xanthus* to identify the leading pole. At the leading pole, RomX is recruited in a RomR-dependent manner, forming the RomRX complex with MglA guanine nucleotide exchange activity. MglA-GDP is diffuse in the cytoplasm, but following conversion of MglA-GDP to MglA-GTP by RomRX, RomRX binds MglA-GTP and sequesters it at the leading pole (Figure 1.1 **a**). RomRX-MglA-GTP stimulates the assembly of, and is incorporated into, the FAC described above (Figure 1.1 **b**).

The FAC moves from the leading to the lagging pole (Figure 1.1 **d**) and then must disassemble. The identity of the lagging pole and the mechanism of FAC disassembly depend on MglB. Once the FAC has moved from the leading to the lagging pole, MglA-GTP encounters MglB and gets converted to MglA-GDP resulting in disassembly of the FAC (Figure 1.1 **e-f**) (Keilberg et al., 2012)(Leonardy et al., 2007). Because RomRX also moves with the FAC towards the lagging pole, a steady accumulation of lagging-pole RomRX builds up over time. Interestingly, although RomR is known to be required for MglA leading pole localisation, MglA continues to localise to the leading pole after most of the RomRX proteins have moved to the lagging pole via the FACs. This observation suggests that there are other polar MglA proteins present that ensures MglA can continue to be recycled. MglB colocalises with RomR at both poles, but the majority of MglB resides at the lagging pole (Keilberg et al., 2012). Thus, both the leading (MglA) and lagging (MglB) cell pole identifiers rely on the RomR protein for cell pole localisation.

Controlling the reversals of cell pole identities requires MglA and MglB to change polar localisation. There are two main prerequisites for this reversal. First, the RomRX proteins must have accumulated at the lagging pole. The time of RomRX accumulation at the lagging pole thus defines a minimum time required for pole reversal. Second, provided enough RomRX protein has accumulated at the lagging pole, switching of pole identity is initiated by the Frz chemotaxis sys-

tem (Ward, Zusman, 1997). Any mutation of the *frz* genes thus result in reversal-deficient phenotypes, with cells gliding continuously (Herrou, Mignot, 2020). These mutant cells are only capable of forming fruiting bodies and a characteristic rippling behaviour, clearly illustrating how the ability to reverse directly alters the macroscopic behaviour of the cells (Herrou, Mignot, 2020).

The Frz system is homologous of the methyl-accepting chemotaxis protein (MCP) chemotaxis system, a system found in most bacteria and archaea (Briegel et al., 2015). The canonical MCP chemotaxis system senses the environmental cues through membrane-associated MCPs. MCPs usually have a sensing domain in the periplasm, such as a Per-Arnt-Sim domain, a transmembrane domain in the CM, and a coiled-coil structure that protrudes into the cytoplasm. The cytoplasmic structure contains a domain often found in histidine kinases, adenylate cyclases, and methyl-accepting proteins, as well as a methylation helix bundle, a flexible bundle, and a signalling domain at the tip of the protein (Bi, Sourjik, 2018). These proteins form large hexagonal arrays with a baseplate at the signalling domain in the cytoplasm consisting of CheW and CheW-like coupling proteins (Briegel et al., 2009). The histidine kinase CheA contains a CheW-like domain and is incorporated into the hexagonal arrays. Upon MCP sensing of environmental signals, the MCP domains undergo a structural rearrangement. This rearrangement is sensed by CheA proteins, which autophosphorylate and transfer the phosphate group onto

a response-regulator protein, CheY (Bi, Sourjik, 2018). In systems governing flagellum chemotaxis, CheY then diffuses to the flagellum basal body and binds to the target proteins, FliM and FliN, resulting in reversal of rotation (Minamino et al., 2019). Tuning of the signalling response is achieved by MCP methylation, performed by the methylesterase CheB and the methyltransferase CheR. CheR methylates MCPs in the "off" state whereas CheB demethylates MCPs in the "on" state leading to an increase or decrease in CheA activity, respectively (Colin, Sourjik, 2017).

There are many variations of the MCP chemotaxis systems, some of which do not influence motility at all, and it is not uncommon to find multiple chemotaxis systems in a single species. For example, in *V. cholerae* there are three MCP chemotaxis systems, each of which forms a separate hexagonal array. Two of these arrays are membrane-bound with different distances from the CM to the signalling domain, and the third is a cytoplasmic array formed by an uncommon MCP (Ortega et al., 2020). Cytoplasmic MCP system arrays are also observed in *Rhodobacter sphaeroides* and the archaea *Methanoregula formicicia* (Briegel et al., 2014)(Briegel et al., 2015). Chemotaxis genes in *M. xanthus* have been duplicated more than what should be expected for the genome size, resulting in a remarkable 8 MCP chemotaxis systems (Che1-8) (Goldman et al., 2006) (Zusman et al., 2007). Che1, also known as Frz, controls reversal rates and pole switching as mentioned above and described

in detail below. The remaining Che operons have roles in a diverse set of cellular functions including motility that is outside the scope of this thesis (Kirby, Zusman, 2003)(Willett, Kirby, 2011)(Vlamakis et al., 2004)(Zusman et al., 2007)(Zusman et al., 2007)(Darnell et al., 2014). The Frz receptors FrzCD are nucleoid-associated and can react to both repellants and attractants. They signal the gliding machine to reverse via two response regulators, FrzZ and FrzX. When environmental cues are picked up by FrzCD, the histidine kinase FrzE autophosphorylates and then transfers the phosphate group to FrzZ and FrzX (Inclan et al., 2007)(Guzzo et al., 2018). Phosphorylated FrzX initiates reversal at the lagging pole provided that sufficient amounts of RomRX have reached the lagging pole. Phosphorylated FrzZ accelerates the release of MglA-GTP from the leading cell pole, although this can also happen in a $\Delta frzZ$ mutant suggesting that FrzZ is not the only component involved in this aspect (Guzzo et al., 2018).

In summary, two main signals are needed for gliding motility reversal. First, sufficient accumulation of RomRX at the lagging pole and, second, the activation of FrzZX by the Frz system. The time it takes RomR to accumulate at the lagging pole acts as a period in which reversals are not possible, limiting the maximum frequency of reversals.

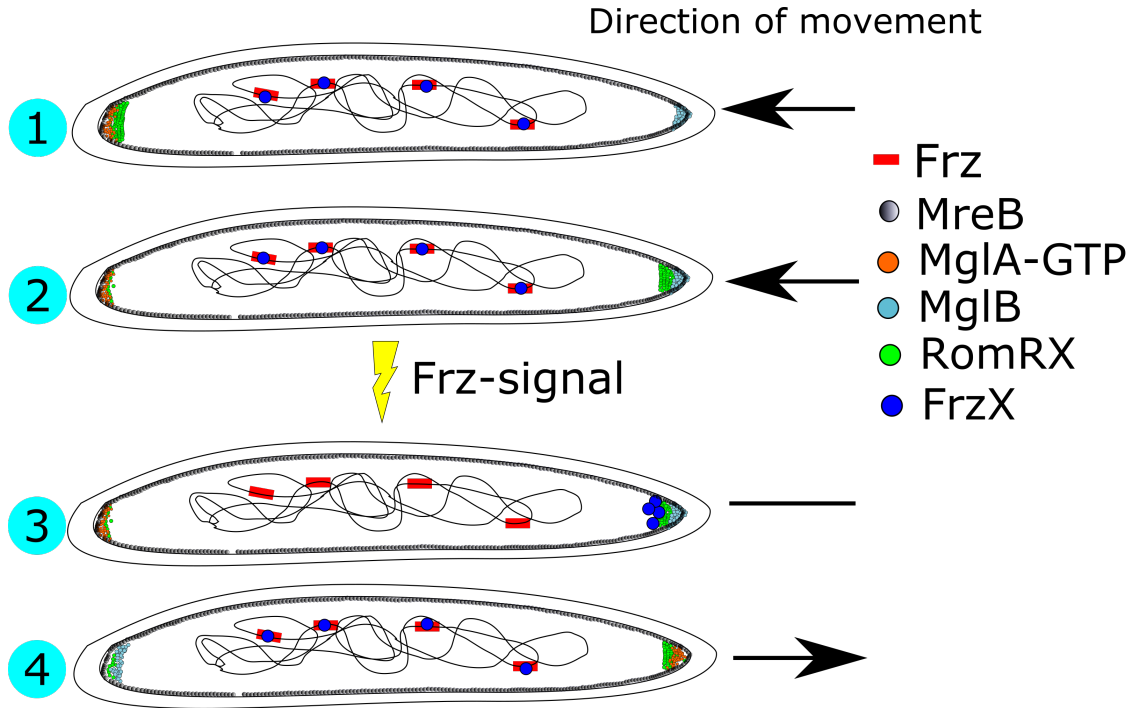


Figure 1.2: Model of *M. xanthus* Frz induced reversal. (1→2) The FACs carry RomRX from the leading to the lagging pole. The steady accumulation of RomRX prepares the cell for reversal (2→3). When nucleoid-associated Frz chemotaxis arrays perceive a stimulus, the response regulator FrzX-phosphate accumulates at the lagging pole. (3→4) By an unknown mechanism, this leads to the release of MglA-GTP from the leading pole, which moves to the old lagging pole and colocalises with MglB for a short period, in which the cells are immobile before MglB moves towards the old leading pole. This switch results in a reversal of the leading and lagging pole, and the cells move towards the new leading pole.

1.0.2 *Flavobacterium johnsoniae* motility

A large proportion of the bacteria in the *Bacteroidetes* family possess the ability to glide over surfaces. Studies on *Bacteroidetes* gliding have been focused on the model organism *Flavobacterium johnsoniae* due to its rapid gliding, ease of culti-

vation, and the availability of tools for its genetic manipulation (McBride, Kempf, 1996)(Rhodes et al., 2011b) (Johnson et al., 2017)(Hahnke et al., 2016). *F. johnsoniae* glides with a speed of $2 \frac{\mu m}{s}$ which is around ~ 25 times faster than *M. xanthus*.

In *Bacteroidetes* gliding, part of the proteins required for gliding are also core components of the type 9 secretion system (T9SS). This secretion system exports adhesin proteins which attach to the substratum and are propelled in a helix along the cell body. There are non-motile *Bacteroidetes* species, such as the dental pathogen *Porphyromonas gingivalis* whose genome encodes the T9SS as well. Thus, the T9SS is not exclusively a component of gliding motility. In *P. gingivalis*, the T9SS is used to secrete a range of pathogenic effector proteins and thus plays an important role in the ability of the organism to cause disease. Nonetheless, the T9SS is absolutely required for gliding motility in motile bacteria. Thus, to understand gliding motility, a thorough review of the T9SS system is required before describing the gliding specific proteins.

The type 9 secretion system

The T9SS secretes folded proteins across the outer membrane (OM) in a PMF-dependent manner (Hennell James et al., 2021). Although detailed mechanistic knowledge of the T9SS is still sparse, most proteins involved are likely to have been

identified. Much of our knowledge of the T9SS has come from studies of the dental pathogen *P. gingivalis* which does not glide. Many of the T9SS components have been given different names in *F. johnsoniae* and *P. gingivalis*. To avoid confusion I will use the *F. johnsoniae* nomenclature but add a *Pg* prefix when referring to the *P. gingivalis* components. The PMF used to drive Type 9 protein export is harnessed by the CM proteins GldL and GldM (Hennell James et al., 2021). These two proteins form a structure reminiscent of the MotAB (*E. coli* nomenclature) stator complexes that harness ion motive force and rotate to power the rotation of the flagellum and the AlgRQS proteins that drive gliding motility in *M. xanthus* (above). Nevertheless, GldLM lack obvious sequence homology to these other proteins and GldM is not thought to bind peptidoglycan to immobilise the motor like MotB does in the MotAB complex. Instead, GldM is hypothesised to rotate, with GldL immobilised by interaction with an unknown large cytoplasmic component via its relatively large (156 residues) cytoplasmic domain. GldLM forms a GldL₅GldM₂ assembly in which the GldL pentamer forms an asymmetric cage around a GldM dimer that rotates as a result of proton flow (Hennell James et al., 2021). Rotating GldM forms a 20 nm diameter ring at the tip that points toward the OM (Hennell James et al., 2021). Rotating GldM is thought to communicate the energy harnessed at the CM to the GldK-GldN complex anchored to the inner side of the OM. *Pg*GldKN form a large ring of approximately 50 nm in diameter

(Gorasia et al., 2016)(Gorasia et al., 2020). *PgGldM* has been shown to interact with *PgGldK* and *PgGldN* in bacterial two-hybrid assays (Leone et al., 2018). From the GldK-GldN ring, the energy is ultimately transferred to the OM Type 9 protein translocon centred on the large β -barrel pore, SprA, via the protein SprE, as suggested by SprE-SprA interaction (Gorasia et al., 2020)(F. Lauber, personal communication). In addition to *gldN*, the *F. johnsoniae* genome contains a partially redundant paralog, *gldO* (Rhodes et al., 2010).

Substrates secreted by the T9SS carry a conserved C-terminal β -sandwich domain (CTD) that mediates targeting to the translocon (Seers et al., 2006)(Veith et al., 2013)(Diego de et al., 2016). The CTDs are divided into two different classes termed type A and type B. Type A CTDs belong to the TIGR04183 domain family and require the OM protein PorV for secretion. (Kharade, McBride, 2015)(Kulkarni et al., 2017). Type B CTDs belong to the TIGR04131 family and are often encoded within the same operon as PorP-like OM proteins (Kulkarni et al., 2019). PorP-like proteins aid in the secretion of their dedicated substrates and seem to replace the role of PorV for Type A CTD containing proteins (Kulkarni et al., 2019). However, some Type B CTD-containing proteins have been reported to be absent from the secretome of a *porV* mutant suggesting that we do not fully understand the role of the CTD sequence in directing secretion (Kharade, McBride, 2015).

In *P. gingivalis*, after substrate proteins have been secreted through the T9SS

they associate with an 'attachment complex', consisting of PorV, the sortase PorU, and the OM proteins PorQ and PorZ. The attachment complex proteolytically removes the substrate CTD and replaces it with anionic lipopolysaccharide (A-LPS) (Glew et al., 2012)(Veith et al., 2013)(Glew et al., 2017). A-LPS is one of two forms of lipopolysaccharides in *P. gingivalis* which differ in the repeating polysaccharide unit (Paramonov et al., 2005)(Paramonov et al., 2001). Attachment of A-LPS anchors the protein to the OM. In *F. johnsoniae*, the role of the attachment complex is not clear as a *porU* deletion does not display any obvious phenotype (Kharade, McBride, 2015). The CTDs of secreted proteins appear to be processed by another route (Mingjun Xu, personal communication), whilst the cell surface adhesins SprB and RemA are probably anchored to the cell surface by the PorP-homologue SprF and PorV, respectively, rather than covalent attachment to LPS as described above (Nelson et al., 2008)(Shrivastava et al., 2012)(Kulkarni et al., 2019).

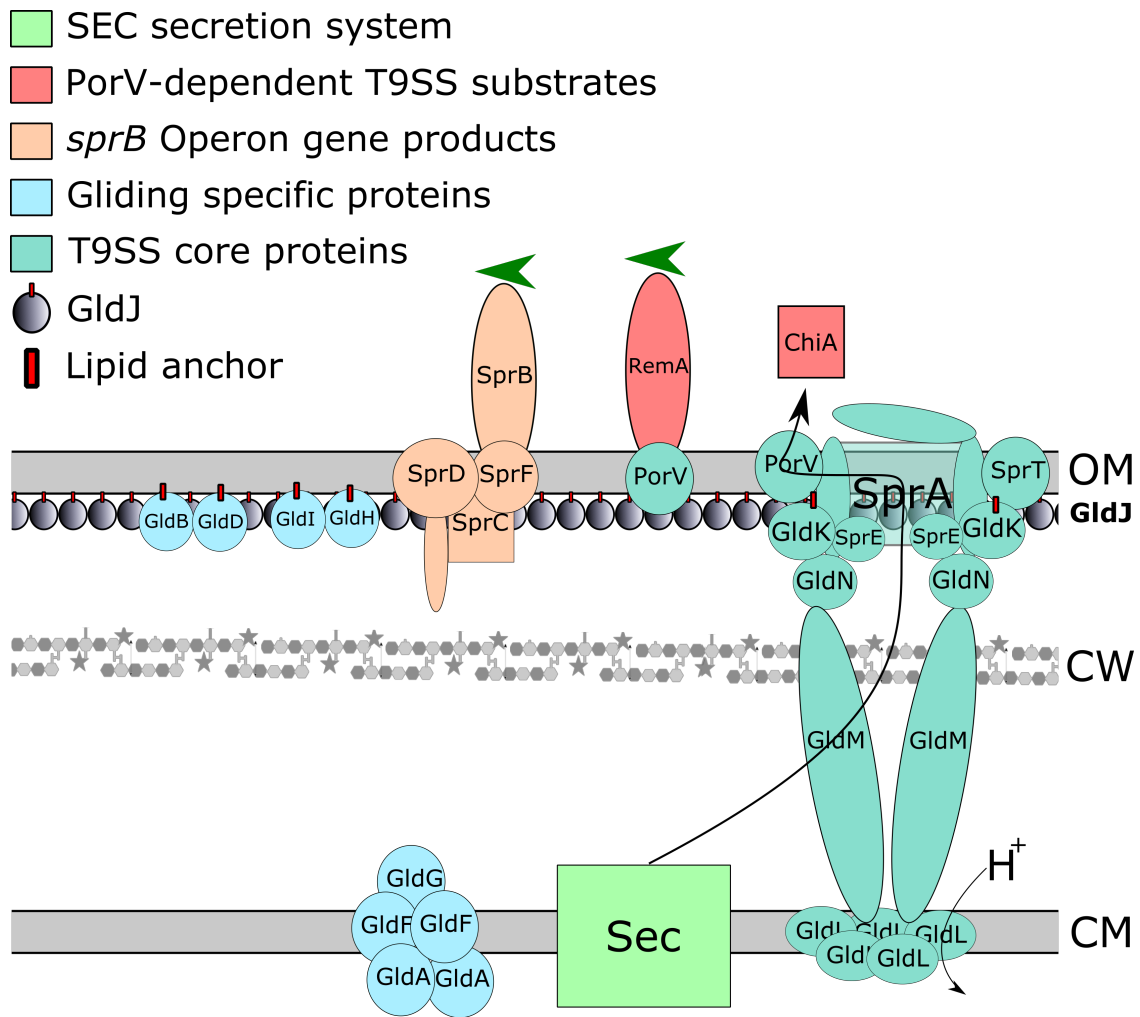


Figure 1.3: A simplified model of the T9SS and associated gliding motility proteins in *F. johnsoniae*. The Sec secretion system transports T9SS substrates such as ChiA, RemA, and SprB across the CM into the periplasm. ChiA is soluble, whereas SprB and RemA are surface-attached adhesins that are propelled along the surface (dark green arrows). The GldL-GldM complex harness the proton gradient, generating energy for the secretion of substrates across the OM. T9SS substrates enter the SprA barrel and are recognised through their C-terminal domain by PorV (or PorP family proteins like SprF) that associate with SprA. The secretion pathway of the ChiA substrate is indicated with a long black arrow. The substrate proteins are then either processed by unknown proteases to remove their C-terminal domain or remain associated at the cell surface with PorV (e.g. RemA) or SprF (SprB). However, direct experimental evidence for this interaction is missing. The function of the conserved β -barrel protein, SprT, is currently unknown, but it is required for T9SS-mediated secretion in *P. gingivalis*. The gliding specific proteins GldAFG form an ABC transporter in the CM, GldDBHI are membrane-associated lipoproteins, arbitrarily assigned to the OM in this figure (McBride, Braun, 2004)(McBride et al., 2003)(Hunnicut, McBride, 2000b)(Hunnicut, McBride, 2001). SprCD are required for SprB-dependent gliding motility and are encoded within the *sprB* operon. GldJ is a lipoprotein assumed to form a helical track at the periplasmic side of the OM(Braun, McBride, 2005). CM, cytoplasmic membrane. CW, cell wall. OM, outer membrane.

Gliding specific proteins

In addition to the proteins involved in T9SS mediated secretion, several gliding-specific proteins have been identified in *F. johnsoniae* through the analysis of gliding-defective mutants. Such mutants can be readily identified because spreading colonies of motile cells form flat, irregular colonies with a lobate edge on solid agar, whereas non-spreading colonies of non-motile mutants form small, round, convex colonies with a smooth edge. The gliding-specific proteins identified in this way were the ATP-binding cassette (ABC) transporter GldAFG and the lipoproteins GldBDHIJ (Agarwal et al., 1997)(Hunnicut et al., 2002)(Braun, McBride,

2005)(Hunnicut, McBride, 2001)(Hunnicut, McBride, 2000a)(McBride, Braun, 2004)(McBride et al., 2003). In addition to these proteins, the major surface adhesin SprB is required for spreading on agar as are a number of proteins encoded in the sprB operon.

GldAFG GldAFG form an ABC transporter. ABC transporters are found in all kingdoms of life. Eukaryotic ABC transporters are most often exporters whereas bacterial ABC transporters perform either import or export functions. The canonical structure of ABC transporters is two nucleotide-binding-domains and two transmembrane domains, sometimes with an accessory ligand-binding protein (Rees et al., 2009). In eukaryotes, this 4-domain complex usually consists of a single polypeptide chain, whereas in bacteria it is often split into several proteins (Rees et al., 2009). In the GldAFG system, GldA has the conserved ATP-binding site. It is found at the CM, despite lacking transmembrane domains. GldA membrane localisation depends on GldFG, suggesting GldA interacts with one or both of these proteins (Hunnicut et al., 2002). GldF contains 6 transmembrane helices and is homologous to the transmembrane domain of other ABC transporters (Hunnicut et al., 2002). GldG contains an N-terminal Sec signal peptide and a C-terminal transmembrane domain (Hunnicut et al., 2002). GldG is homologous to other ABC transporter accessory proteins, thus it is assumed that the core ABC transporter has the composition 2 GldA₂GldF₂GldG₁ or GldA₂GldF₂GldG₂. Disruption

of any one of *gldAFG* by either deletion or point mutations in the predicted ATPase site of GldA leads to loss of motility and secretion (Johnston et al., 2018)(Hunnicut et al., 2002)(Agarwal et al., 1997). However, some gliding species do not possess *gldAFG* suggesting the transporter may not be an integral part of the gliding machinery (McBride, Zhu, 2013). Consistent with this idea, a transposon screen for gliding motility mutants in *Cellulophaga algicola* failed to identify an ABC transporter (Zhu, McBride, 2016).

GldHIJ GldDHIJ are all lipoproteins that are required for gliding motility in *F. johnsoniae* (McBride et al., 2003) (McBride, Braun, 2004).

Deletion of *gldD* results in non-motile *F. johnsoniae*, but deletion of the *C. algicola* *gldD* ortholog, results in partially reduced colony-spreading (Zhu, McBride, 2016)(Hunnicut, McBride, 2001). *gldD* is mostly found in gliding, and rarely in immobile, *Bacteroidetes* species (McBride, Zhu, 2013).

A gliding deficient *gldB* mutant with a single residue mutation is partially recovered through the overexpression of *gldE* (Hunnicut, McBride, 2001). *gldB* is found in most *Bacteroidetes* species and may have a function that is not exclusive to gliding motility (McBride, Zhu, 2013)

GldH and GldI are found in both gliding and non-gliding *Bacteroidetes* (McBride, Zhu, 2013). *gldH* is cotranscribed with *fjoh_0891* that encodes a PSP1-domain containing protein. The operon organisation of *fjoh_0891-gldH* is conserved in

other *Bacteroidetes* but *fjoh_0891* has not been identified in transposon mutagenesis screens or been deleted to assess its role in gliding motility (McBride et al., 2003). GldI is a predicted peptidyl-prolyl cis/trans-isomerase (PPI) involved in protein folding and the two genes immediately downstream of *gldI* are also encoding predicted PPI proteins (McBride, Braun, 2004).

GldJ is a protein with a helical subcellular organisation (Braun, McBride, 2005). GldJ is homologous to GldK and both proteins have a fold that is related to sulfatase-modifying enzymes (Braun et al., 2005). *gldABDFHI* mutants all result in destabilisation and loss of the GldJ protein, the absence of which in turn destabilises GldK, resulting in the loss of both Type 9 secretion and gliding (Johnston et al., 2018)(Braun, McBride, 2005). Although GldJ is required for GldK stability, deletion of any one of *gldKLMN* does not affect the accumulation of GldJ (Johnston et al., 2018)(Shrivastava et al., 2013). The C-terminal domain of GldJ is conserved amongst GldJ proteins in *Bacteroidetes* and contains several positively charged amino acids (Johnston et al., 2018). Although *gldJ* cannot be deleted without disrupting GldK, a truncated version of GldJ lacking the C-terminal 13 amino acids (GldJ₅₄₈) resulted in wild-type levels of GldK protein and a functional T9SS. However, cells producing GldJ₅₄₈ were unable to support gliding or helical movement of the SprB adhesins (Johnston et al., 2018). In light of these results, it has been proposed that GldJ might be a track-forming, or track-associated, protein,

helping to guide the helical movement of the adhesins around the cell body while connecting with the T9SS to harness energy (Shrivastava, Berg, 2020).

GldCE The role of GldC or GldE in gliding motility is not clear.

GldE is a predicted CM protein with 3 N-terminal transmembrane helices, two Cystathione β -synthase domains forming a globular fold often found in CorC/HlyC $\text{Mg}^{2+}/\text{Co}^{2+}$ transporter proteins, and a C-terminal flavin adenine dinucleotide-binding domain belonging to the SSF56176 superfamily. GldE has, so far, not been deleted in *F. johnsoniae*. The only evidence for the role of GldE in gliding is that ~ 10 -fold overproduction of GldE was shown to partially restore the gliding ability of a *gldB* point mutant which was otherwise void of motility (Hunnicut, McBride, 2001), and the synteny of *gldE* with another gliding gene, *gldD*.

gldC forms an operon with *gldB* which encodes a soluble predicted cytoplasmic protein (Hunnicut, McBride, 2000a)(McBride et al., 2003). Although GldB is required for T9SS activity and gliding motility, *gldC* can be deleted without complete loss of motility (Hunnicut, McBride, 2000a).

The *sprB* operon The main adhesin in *F. johnsoniae*, SprB, is a Type B CTD containing T9SS substrate that forms 150nm long filaments on the cell surface (Rhodes et al., 2011a). SprB moves in a helix around the cell body (Nakane et al., 2013). The gene encoding SprB is part of an operon containing the genes

sprBCDEF, *remFG*, and *fjoh_0982*. Similar to the *gld* genes described above, mutants of most of the genes of the *sprB* operon form non-spreading colonies on PY2 agar plates. However, in contrast to the *gld* genes, these *sprB* operon genes are not required for T9SS secretion.

SprB is secreted through the T9SS and localises to the cell surface. The secretion, and possibly cell-surface attachment, is aided by the *sprB* operon protein SprF. SprF is an OM β -barrel of the PorP family. It is required for the secretion of SprB, but not for secretion of PorV-dependent substrates (Rhodes et al., 2011a). SprF localises to the OM where it is likely to interact with SprB post-secretion (Kulkarni et al., 2019).

The *F. johnsoniae* genome encodes 9 paralogs of *sprF* outside the *sprB* operon. These paralogs are often encoded next to *porE* (*PGN_1296*)- and *sprB*-like genes (table 1.2) (Naito et al., 2019). Exchanging SprF for one of its paralogs (Fjoh_3951) did not yield successful secretion of SprB and exchanging SprB for the SprB-paralog Fjoh_3952 did not result in SprF-mediated secretion of Fjoh_3952, suggesting that the PorP-like proteins are specific to the Type B CTD encoding genes in the same operon (Kulkarni et al., 2019).

Table 1.2: Summary of the genetic linkage of *porP* family genes to *porE*- and *sprB*-like genes in *F. johnsoniae*.

<i>porP</i> homolog	genetically linked <i>porE</i> homolog	Genetically linked <i>sprB</i> homolog	<i>sprB</i> homolog product size
<i>sprF fjoh_0978</i>		<i>sprB fjoh_0979</i>	6497aa/672kDa
<i>sprD fjoh_0980</i>		<i>sprB fjoh_0979</i>	6497aa/672kDa
<i>fjoh_1646</i>	<i>fjoh_1647</i>	<i>fjoh_1645</i>	2487aa/258kDa
<i>fjoh_1677</i>			
<i>fjoh_1986</i>		<i>fjoh_1985</i>	2064aa/221kDa
<i>fjoh_2274</i>	<i>fjoh_2275</i>		
<i>fjoh_3477</i>	<i>fjoh_3476</i>	<i>fjoh_3478</i>	3737aa/386kDa
<i>fjoh_3951</i>	<i>fjoh_3950</i>	<i>fjoh_3952</i>	3190aa/331kDa
<i>fjoh_3972</i>	<i>fjoh_3973</i>	<i>fjoh_3971</i>	3602aa/375kDa
<i>fjoh_4539</i>	<i>fjoh_4540</i>	<i>fjoh_4538</i>	2588aa/271kDa
<i>fjoh_4749</i>		<i>fjoh_4750</i>	1463aa/158kDa

Both *sprC* and *sprD* are required for gliding on agar. However, mutants lacking either of the two genes are still able to produce helically moving SprB on the cell surface (Rhodes et al., 2011a). When both mutations are combined it is reported that the resulting strain is no longer able to support the helical movement of SprB molecules even though SprB is still localised to the cell surface (Kulkarni et al., 2019).

remF and *remG* both have paralogs (*remH* and *remI*, respectively) in the *F. johnsoniae*

genome. Deletion of the paralog pairs *remFH*, or *remGI*, abolishes colony spreading similar to the deletion of *sprBCDF* (Rhodes et al., 2011b).

In addition to *sprCDBF* and *remFG*, the *sprB* operon contains another gene, *fjoh_0982*, however it does not seem to be involved in gliding and no other gliding species encode a homolog of this gene (Rhodes et al., 2011a).

The cell surface lectin adhesin RemA RemA and Fjoh_0803-0806 are paralogous cell surface adhesins. RemA has a lectin-binding domain and promotes cell-cell aggregation by adhering to polysaccharides secreted by *F. johnsoniae* (Shrivastava et al., 2012). RemA moves in a helical track similar to SprB, but, unlike SprB, RemA is dependent on PorV for secretion (Kharade, McBride, 2015). Both SprB and RemA can mediate attachment to glass slides but only SprB supports colony spreading on agar plates (Shrivastava et al., 2012).

In summary, the gliding motility system in *F. johnsoniae* and more broad, in gliding *Bacteroidetes*, is composed of a multitude of components (See Table 1.3). This large number of proteins requires a complex organisation and spatiotemporal control, to provide the cells with the ability to move with speeds in the $\frac{\mu m}{s}$ range. To get a glimpse of this macromolecular masterpiece in action, I will next review the current hypotheses that attempt to explain the physical basis of gliding motility in *Bacteroidetes*, using *F. johnsoniae* as a model.

Table 1.3: Summary of *F. johnsoniae* motility proteins and their subcellular localisation. CM, Cytoplasmic Membrane. P, Periplasm. OM, Outer membrane. Gliding indicates genes required only for gliding motility. T9SS indicates genes that make up the type 9 secretion system.

Protein Name	Locus Tag	Protein ID	Predicted Localisation	gene Group
<i>gldA</i>	<i>fjoh_1516</i>	A5FJR5	CM	Gliding
<i>gldB</i>	<i>fjoh_1793</i>	A5FJ02	OM or CM	Gliding
<i>gldC</i>	<i>fjoh_1794</i>	A5FIY5	CM	Gliding
<i>gldD</i>	<i>fjoh_1540</i>	A5FJP0	OM or CM	Gliding
<i>gldE</i>	<i>fjoh_1539</i>	A5FJQ6	CM	Gliding
<i>gldF</i>	<i>fjoh_2722</i>	A5FGC8	CM	Gliding
<i>gldG</i>	<i>fjoh_2721</i>	A5FGC7	CM	Gliding
<i>gldH</i>	<i>fjoh_0890</i>	Q8KRP0	OM or CM	Gliding
<i>gldI</i>	<i>fjoh_2369</i>	A5FHC1	OM or CM	Gliding
<i>gldJ</i>	<i>fjoh_1557</i>	A5FJM9	OM	Gliding
<i>gldK</i>	<i>fjoh_1853</i>	A5FIS8	OM	T9SS
<i>gldL</i>	<i>fjoh_1854</i>	A5FIS9	CM	T9SS
<i>gldM</i>	<i>fjoh_1855</i>	A5FIT0	CM and P	T9SS
<i>gldN</i>	<i>fjoh_1856</i>	A5FIT1	OM	T9SS
<i>gldO</i>	<i>fjoh_1857</i>	A5FIT2	OM	T9SS
<i>sprA</i>	<i>fjoh_1653</i>	Q5I6C7	OM	T9SS
<i>sprB</i>	<i>fjoh_0979</i>	A1E5U5	OM	Gliding

Protein Name	Locus Tag	Protein ID	Predicted Localisation	gene Group
<i>sprC</i>	<i>fjoh_0981</i>	A1E5U3	P	Gliding
<i>sprD</i>	<i>fjoh_0980</i>	A1E5U4	OM	Gliding
<i>sprE</i>	<i>fjoh_1051</i>	A1E5T9	P	T9SS
<i>sprF</i>	<i>fjoh_0978</i>	A5FLA7	OM	Gliding
<i>sprT</i>	<i>fjoh_1466</i>	A5FJX0	OM	T9SS
<i>remF</i>	<i>fjoh_0984</i>	A5FL98	P	Gliding
<i>remG</i>	<i>fjoh_0983</i>	A5FL97	P	Gliding

The physical basis of gliding

F. johnsoniae cells move with a corkscrewing motion, mediated by the helical movement of SprB and other adhesins. Nakane et al. (2013) found that SprB molecules move with the same apparent pitch and speed in both directions along, and relative to, the cell body. This prompted them to suggest that the adhesins move on a track that forms a helical loop. In this helical loop model, it is proposed that SprB binds to the substratum causing the cell to roll relative to the adhered SprB protein until it reaches the rear of the cell. At the rear of the cell, SprB detaches from the substratum and moves toward the front of the cell without attaching to the substratum before reaching the front of the cell again (see Figure 1.4). It is currently unknown how *F. johnsoniae* controls attachment/detachment of SprB.

For SprB to move in a helix around the cell body, energy must be provided and the movement direction controlled. The energy is generated at the CM by the T9SS. Components of the T9SS are located at distinct foci around the cell (Lauber et al., 2018)(Shrivastava, Berg, 2020). If the energy is harnessed at these discrete positions, it requires a way of transferring the energy from the T9SS motor to the adhesins that move in a continuous helical path.

One proposed way is a track, providing both a means for energy transfer to the adhesins and a path for the adhesins to follow. Whether the track itself moves is unclear. The most likely candidate for the track is GldJ. As described above, the majority of gliding-specific proteins conserved in all gliding *Bacteroidetes* are required for GldJ stability, and GldJ has a helical distribution within the periplasm, and it is anchored to the membrane via a lipid-tail. In accordance with the role of GldJ as a track protein, a 13 residue C-terminal truncation of GldJ results in the cell surface SprB adhesins being immobile (Johnston et al., 2018).

It is still unclear how the energy is transferred from the T9SS to the track, and further to SprB. It has been suggested that gliding is powered by a rotary motor based on the observation that cells rotated around fixed points at a frequency of ~ 1 Hz when attached at a single point to glass slides via SprB antisera (Shrivastava et al., 2015). It was claimed that the point of fixation corresponded to the localisation of GldL molecules suggesting the T9SS and SprB both localise to the point of fixation in these experiments (Shrivastava, Berg, 2020). Based on these results, Shrivastava, Berg (2020) suggested a rack and pinion model wherein the T9SS is a rotating pinion that drives a moving rack with attached

SprB adhesins along a fixed track around the cell. In the 'rack and pinion' model, the rotary motor has a drive shaft whose radius is a determining factor in the speed by which the adhesins can be propelled. Shrivastava, Berg (2020) calculated the radius to be ~ 80 nm, which would result in $1.5 \frac{\mu m}{s}$ given that the motor rotated at 3 Hz (estimated max frequency of rotation). Because the shaft must engage with the track (GldJ), it must be located near the OM. The GldK lipoprotein is homologous to GldJ and is likely to form the shaft. GldKN are known to form rings in *P. gingivalis* near the OM. These rings are only 50 nm in diameter, however. *P. gingivalis* is unable to glide, so it is possible that the GldKN proteins in *F. johnsoniae* form either a larger ring or an altogether different structure. If such a large energised ring exists in *F. johnsoniae*, another question becomes how the 20 nm diameter ring of rotating GldM proteins can transfer energy and cause a much larger ring to rotate. There is currently no knowledge of how this organisation is achieved, and so it is still difficult to judge the exact structure of this assembly.

In summary, the current model for *F. johnsoniae* gliding motility is a rack-and-pinion model wherein helically moving SprB adhesins are attached to a moving rack that follows a defined helical path (Figure 1.4). The rack (and attached adhesins) is moved along by the power generated by rotary T9SS complexes distributed around the cells in distinct foci. As the SprB molecules move from the leading to the lagging pole, they adhere to the substratum, but not while they move back towards the leading pole. Evidence for attachment/detachment or the regulation thereof is still missing.

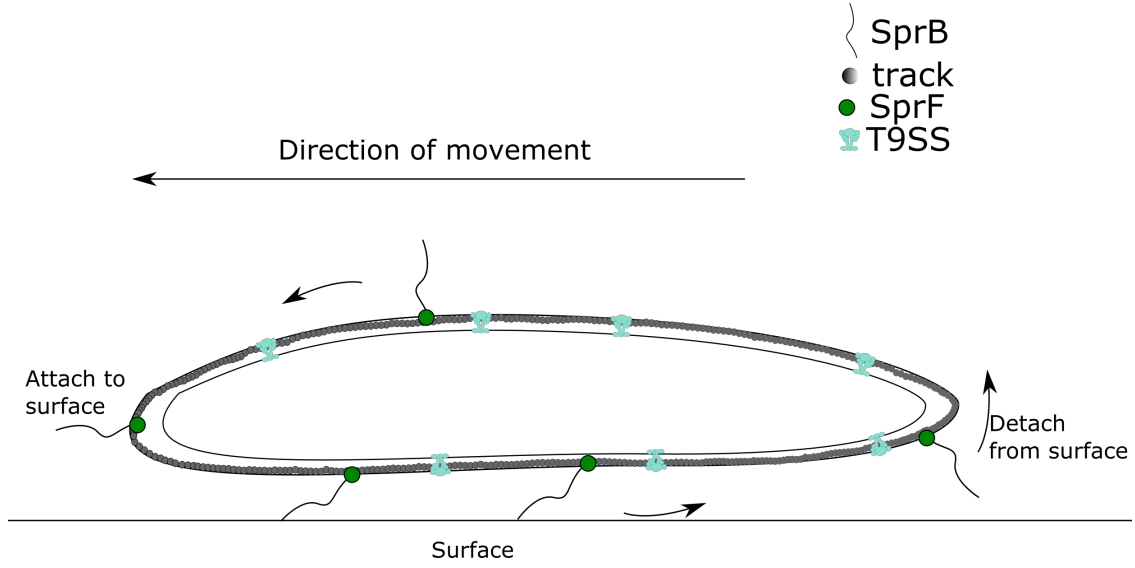


Figure 1.4: Model of *F. johnsoniae* gliding motility. SprB, assumed to be in complex with SprF, moves in a left-handed helix around the cell body along a defined track. The SprB proteins are thought to be engaged with a moving rack (GldJ) that moves along a defined path by the power provided by discrete T9SS complexes. SprB is hypothesised to only bind the surface as it moves from the leading pole toward the lagging pole. As SprB reaches the lagging pole, it detaches and continues moving in a non-binding state toward the leading pole where it will then re-engage with the surface and repeat the cycle.

1.0.3 Comparison of *M. xanthus* and *F. johnsoniae* gliding motility

Although there is no sequence homology between the gliding motility proteins of *M. xanthus* and *F. johnsoniae*, both systems share several functional aspects and are facing some of the same challenges (Wadhwa, Berg, 2021). Gliding motility in both *M. xanthus* and *F. johnsoniae* can be observed as a forward rolling of the cell body with points of fixation to the surface on which the bacteria move. One striking difference between the two types of motility is the speed (Table 1.4). *F. johnsoniae* glide with a speed of $\sim 2 \frac{\mu\text{m}}{\text{s}}$

whereas *M. xanthus* only glide with a speed of $\sim 4 \frac{\mu m}{m}$ (Wadhwa, Berg, 2021). Why are two systems that seemingly evolved a mechanically similar mode of movement 25-fold different in speed? Some answers may be found in the molecular arrangement of gliding proteins and likely reflect the different solutions that have evolved to address fundamental limitations of the motility systems.

As discussed in the sections above, both *M. xanthus* and *F. johnsoniae* propel adhesins attached to the substratum around in a helix to achieve net cell movement (Table 1.4). A fundamental limit for both these systems must therefore be to detach from the substratum. Detachment is required to move further since an adhesive complex that remains attached would immobilise the cell. In *F. johnsoniae* the adhesins continue to move in both directions of the helix. It is hypothesised that the adhesins let go of the surface and continue in a helical loop towards the front of the cells, managing to avoid binding the surface until it reaches the front of the cell again through an unknown mechanism (Nakane et al., 2013). In *M. xanthus*, FACs are assembled at the leading pole and the FAC-associated adhesin is secreted and attach to the substratum (Islam et al., 2020)(Islam, Mignot, 2015). The FAC move to the lagging pole where it disassembles and the adhesin is cleaved off. During disassembly, most of the FAC components are recycled to the leading pole without generating force, where they reassemble into new FACs while a few (RomRX) remain at the lagging pole and build up there to prime for a future reversal event (Szadkowski et al., 2019). This mechanism explains the differences in speed between the two types of gliding motility. *M. xanthus* must synthesise, secrete, and proteolyse the adhesin with every FAC cycle from leading to lagging cell pole whereas *F. johnsoniae* seem

capable of continuously reusing its adhesins through a dynamic cycle of a still unknown mechanism of attachment/detachment.

Another potential explanation for the difference in speed between the two systems could come from the organisation of the gliding machinery. A fundamental requirement of the helical movement is the ability of the gliding machine to provide direction and energy to power the movement of the adhesins. In both motility systems, the energy comes from the PMF, thus one fundamental limit is that the power must be generated at the CM and connected through the machine to the adhesins (Wadhwa, Berg, 2021). In *M. xanthus*, the motor proteins move helically with the aid of the cytoplasmic MreB track (Table 1.4) (Nan et al., 2011). Since the FAC traverses the envelope, it must be able to pass through the cell wall as it moves from the leading to the lagging pole. Because MreB is known to direct cell-wall synthesis, it could be that the cell wall of *M. xanthus* is organised to allow for this movement, however, the level of remodelling likely poses a time constraint nonetheless.

The *F. johnsoniae* gliding motility machine has overcome the limitation of the cell wall by organising the helical track at the periplasmic side of the OM (Table 1.4). The stationary motor units are thought to harness the PMF at the CM and transfer it to OM lipoproteins that in turn can connect it to the track. Adhesins connected to the track are then via an unknown mechanism pushed along the track. Thus, the flow of energy seems to only pass by the cell wall at the points where T9SS assemblies are located. There has not been identified a protein needed for gliding motility in the cytoplasm, and the addition of the anti-MreB drug A22 does not inhibit gliding (Shrivastava, Berg, 2020).

Although the speed of translocating FACs in *M. xanthus* is slow, AglR and MreB move up to 2.5 $\mu\text{m/s}$ when they are not associated with the FAC. The non-adhering speed of the Agl-MreB complex is very close to the speed of moving SprB molecules and the speed observed for spheres attached to the little-studied gliding motility of *C. aggregans*. Speculatively, although GldLM are likely immobile and transfers the PMF to the adhesins through a number of other proteins, and the motor of *C. aggregans* is unknown, it may be that this maximum speed is a fundamental limit of gliding motility motors. In this case, *F. johnsoniae* approaches that speed by a more efficient method of transferring the energy to the other side of the cell wall.

Both *M. xanthus* and *F. johnsoniae* display chemotactic behaviour. *F. johnsoniae* has been shown to move away from the repellants: H_2O_2 , OCl^- , and N-chlorotaurine (Liu, Fridovich, 1996). *M. xanthus* moves away from isoamyl alcohol and towards a mix of casitone and yeast extract (Shi et al., 1993). *M. xanthus* controls the direction of gliding movement with chemotaxis proteins. Because components of the gliding machine are organised asymmetrically along the cell body, a designated leading and lagging pole is defined. Using the Frz chemotaxis protein signal, *M. xanthus* can activate a reversal event that leads to the switching of the leading and lagging pole by redistributing the asymmetrically localised motility proteins, ultimately causing a 180° reversal of movement. Little is known about *F. johnsoniae* control of direction, in part because there has been no asymmetric distribution observed for any of the known motility proteins. According to the current model of *F. johnsoniae* gliding, there must be such an asymmetric organisation present, however, as the cells would otherwise be incapable of discerning one cell pole

apart from the other. *F. johnsoniae* chemotaxis control is enigmatic, as the genome lacks homologs of the typical chemotaxis proteins: CheA, CheW, and MCPs, suggesting a potentially novel signalling pathway must govern the reversal mechanism.

Table 1.4: Comparison of *M. xanthus* and *F. johnsoniae* gliding motility.

	<i>F. johnsoniae</i>	<i>M. xanthus</i>	Reference
Movement speed avg.	2 $\mu\text{m/s}$	4 $\mu\text{m/min}$	(Nakane et al., 2013)(Wu et al., 2009)
Swarm expansion rate	?	1.6 $\mu\text{m/min}$	(Wu et al., 2009)
Speed of Adhesin	2 $\mu\text{m/s}$	3.2 $\pm$ 0.9 $\mu\text{m/min}$	(Nakane et al., 2013)(Faure et al., 2016)
Speed dependent on cell size	No	No	(Sun et al., 1999)
Reversal frequency	?	8.8 $\pm$ 2.1 min	(Wu et al., 2009)
Pitch angle relative to long cell axis	19.0 $\pm$ 11.5 $^\circ$	78 $\pm$ 5 $^\circ$	(Nakane et al., 2013)(Faure et al., 2016)
Protruding OM filament	Yes	No	(Nakane et al., 2013)

	<i>F. johnsoniae</i>	<i>M. xanthus</i>	Reference
Slime secretion	?	Yes	(Ducret et al., 2012)
PMF as power source	Yes	Yes	(Nakane et al., 2013)(Sun et al., 2011)
Helical loop	Yes	Yes	(Nakane et al., 2013)(Lünsdorf, Schairer, 2001)
Motor moves along track	No?	Yes	(Faure et al., 2016)(Lauber et al., 2018)(Shrivastava, Berg, 2020)
Helical track determinant	GldJ?	MreB	(Fu et al., 2018)
Helical track localisation	Outer Membrane	Cytoplasm	(Fu et al., 2018)

	<i>F. johnsoniae</i>	<i>M. xanthus</i>	Reference
Proteolytic regulation of adhesins	No	Yes	(Islam et al., 2020)(Nakane et al., 2013)
Cell flip to change direction	Yes	Yes	-

1.1 Aims of the thesis

The general aim of this thesis is to obtain molecular-mechanical detail of the *Bacteroidetes* gliding motility machine. Subsequent chapters will address the following specific aims:

- The initial aim of this project is to develop a methodology that allows the visualisation and dynamic of the track topology. Little is known about the dynamics and topology of GldJ. It forms a track which has been visualised only in fixed cells. The images are of too low quality to get a detailed understanding of the organisation, thus a method is needed to study this. Therefore, I will develop a method for 3-dimensional single-particle tracking of SprB that will allow me to determine the dynamics and topology of SprB movement and thereby infer the underlying tracks' topology.
- The second aim of this project is to study if and how the major adhesin, SprB,

controls adhesion. SprB is absolutely required for active gliding under known laboratory conditions. However, it is only theorised that SprB can turn adhesion on and off during movement even though this is a fundamental physical requirement of the current gliding model. The *sprB* operon components have only been studied little and pose a good starting point to further our understanding of the function of SprB. Therefore, I will examine the role of the additional *sprB* operon protein in control of *F. johnsoniae* gliding motility.

- The last aim of this project is to verify experimentally that GldJ interacts with both the T9SS and adhesins, and thereby further study the role of GldJ in gliding motility. GldJ is hypothesised to bridge the energy-supplying T9SS with SprB and other adhesins. This has never been shown, and so I will attempt to verify this hypothesis by purifying GldJ and its interaction partners.

Chapter 2

Materials and methods

2.1 Growth conditions

2.1.1 *E. coli*

E. coli strains were routinely grown at 37 °C in LB medium (Table 2.1) supplemented with appropriate antibiotics as listed in table 2.2. For liquid cultures, 180 rpm shaking was used.

2.1.2 *F. johnsoniae*

F. johnsoniae were routinely grown in casitone yeast extract (CYE, Table 2.1). Where indicated, strains were grown in Motility medium (MM) or Peptone yeast (PY2) medium (Table 2.1). PY2 medium was used during imaging sessions since it provides optimal gliding conditions, and low-to-no autofluorescence. PY2 agar plates were used to assay

colony spreading. Where required, 100 $\mu\text{g}/\text{ml}$ erythromycin was included in the growth medium.

Table 2.1: Media used in this thesis. Agar is only added when pouring agar plates with the respective medium.

LB Agar	
Tryptone	10g/L
Yeast Extract	5g/L
NaCl	10g/L
Agar	20g/L
MYA Agar	
MgSO ₄	0.5 mM
FeSO ₄	0.05 mM
EDTA	0.04 mM
potassium phosphate [pH 7.25]	20 mM
yeast extract	0.1/L
Agar	15g/L
Peptone Yeast (PY2) pH7.3	
peptone	2g/L
yeast extract	0.5g/L
Casitone Yeast Extract (CYE)	
Casitone	10g/L
yeast extract	5g/L

MgSO ₄	8mM
Tris buffer [pH 7.6]	10mM
Motility medium (MM)	
Casitone	3.3g/L
Yeast extract	1.7g/L
Tris buffer [pH 7.5]	3.3mM

Table 2.2: Antibiotics used in this thesis.

Antibiotic	Concentration
Erythromycin	100 μ g/ml
Ampicillin	100 μ g/ml

2.2 Molecular biology

2.2.1 Cloning methods

Polymerase chain reaction

Polymerase chain reaction (PCR) was routinely used to amplify DNA fragments for assembly into plasmids. Primers were purchased at Sigma Aldrich (Merck), and purified by desalting or, if longer than 65 basepairs, polyacrylamide gel electrophoresis (PAGE) purified. The design of primers for PCR is described under the respective cloning sections.

The PCR reaction comprised Q5® High-Fidelity 2X Master Mix (New England Biolabs, deoxyribonucleotide triphosphates, proprietary buffer, Q5 High-Fidelity DNA Polymerase, and Mg⁺⁺) , H₂O, 10 μM of each forward and reverse primer, and template DNA. The PCR program was constructed according to the manufacturer’s recommendation, as laid out in Table 2.3.

Table 2.3: PCR program for Q5 polymerase reactions.

Step	Temperature	Time
Initial Denaturation	98°C	30 s
25–35 Cycles	98°C	15 s
	50–72 °C	10–30 s
	72 °C	30 s/kb
Final Extension	72 °C	2 min
Hold	4 °C	∞

Colony PCR was routinely used to screen colonies, during cloning, for the correct insert or deletion of DNA fragments. Because high-fidelity was not required for this, the cheaper and more error-prone *taq* enzyme was used. The reaction mixture was comprised of the same as for the Q5 reaction mentioned above, except OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs) was used instead of Q5® High-Fidelity 2X Master Mix. The PCR program was also slightly altered, according to the manufacturer’s

recommendations, to accommodate the lower extension temperature under which the enzyme is active, as well as overall lower annealing temperatures (Table 2.4).

Table 2.4: PCR program for OneTaq polymerase reactions.

Step	Temperature	Time
Initial Denaturation	98°C	30 s
30–35 Cycles	98°C	15 s
	45–68 °C	30 s
	68 °C	60 s/kb
Final Extension	68 °C	5 min
Hold	4 °C	∞

Gibson assembly

The majority of plasmids described in this thesis were assembled using Gibson Assembly (Gibson et al., 2009). Gibson Assembly allows for the assembly of multiple DNA fragments with overlapping ends in a single reaction. The three active enzymes involved in this process are: (i) an exonuclease that creates single-stranded ends, (ii) a DNA polymerase to fill in the gaps as two homologous ends anneal, and (iii) a DNA ligase to covalently seal the nicks. The reaction mix was incubated for 4 hours at 50 °C. Plasmids assembled by Gibson Assembly were transformed directly into chemically competent *E. coli* cells

as described in Transformation of *E. coli*. Primers for Gibson Assembly were designed using SnapGene software. In general, primers were designed to produce DNA fragments with 40 bp complementary to the partner DNA fragment. The length of the primer complementary to the template DNA in the PCR reactions was adjusted to reach an annealing temperature of 50-72 degrees, calculated using the New England Biolabs melting temperature calculator (<https://tmcalculator.neb.com/#!/main>).

Site directed mutagenesis

The commercially available technique - Site-directed mutagenesis - was used to introduce or delete short DNA fragments such as those coding for Twin-Strep-tags or His₆-tags, or that result in truncations of proteins. For insertions, primers complementary to the site of insertion were designed with overhangs containing the sequence to be inserted. When the insertion sequence was large, it was split between the forward and reverse primer. For deletions, primers complementary to the template DNA surrounding the area targeted for deletion were designed. Following PCR amplification using Q5 polymerase as described above, the DNA fragment was treated with a mixture of Kinase, Ligase, and DpnI (KLD) for 10 min at room temperature (RT), to seal the two blunt ends of the amplified DNA fragments together. In some cases, the PCR reaction was incubated for 1 hour with DpnI at 50 °C prior to treatment with KLD to ensure sufficient cleavage of methylated template DNA material. The product of the KLD reaction was subsequently transformed into chemically competent *E. coli* cells as described below.

2.2.2 Transformation of *E. coli*

Plasmids prepared as described in 2.2.1 were routinely transformed into chemically competent *E. coli* cells. Chemically competent cells were prepared by growing the required strain overnight (ON) in 5 mL LB medium (Table 2.1) at 37 °C, with 180 rpm shaking. 1 mL of the ON culture was subcultured into 50 mL fresh LB and grown at 37 °C with 180 rpm shaking to an OD₆₀₀ of 0.5. Cells were pelleted by centrifuging at 2500 g, 4 °C for 10 min. The supernatant was discarded, and the cell pellet was resuspended in 15 mL ice-cold TFBI (Table 2.5) and incubated on ice for 2 hours. Following incubation, the cell suspension was centrifuged at 2500g, 4 °C for 10 min and the supernatant was discarded. The cell pellet was resuspended in ice-cold 1.5 mL TFBII (Table 2.5) and immediately aliquoted in 50 μ L and flash-frozen in liquid nitrogen. The competent cells were stored at -80 °C and thawed on ice when used.

Table 2.5: Buffer components for the two buffers used in the creation of chemically competent *E. coli* cells.

TFBI pH 5.8	
RbCl ₂	100mM
potassium acetate	30mM
CaCl ₂	10mM
MnCl ₂	50mM
hexamine cobalt chloride	3mM
glycerol	15% v/v
TFBII pH 6.5	
MOPS	10mM
CaCl ₂	75mM
RbCl ₂	10mM
glycerol	15% v/v

2.2.3 Conjugation of plasmids into *F. johnsoniae*

E. coli S17-1 was used as a donor to conjugate pYT313-derived suicide plasmids into *F. johnsoniae* by biparental mating (Simon et al., 1983). Chromosomal insertions of the

plasmid were selected for using erythromycin containing CYE agar plates (Table 2.1). The resulting strains were grown in CYE without erythromycin to promote spontaneous loss of the plasmid, and plated on CYE plates with 5 % sucrose for SacB dependent counter-selection. The chromosomal modification was confirmed by sequencing.

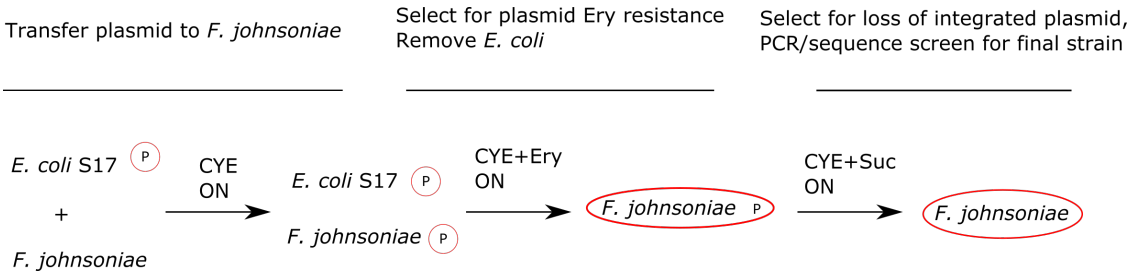


Figure 2.1: Diagram depicting the steps in the conjugation of a pYT313-derived plasmid into *F. johnsoniae*. The donor *E. coli* S17 strain is grown together with a *F. johnsoniae* acceptor strain on CYE plates. The plasmid is transferred to *F. johnsoniae* and the *F. johnsoniae* specific promoter controlling the erythromycin (Ery) resistance gene allows selection of only *F. johnsoniae* strains that carry the plasmid. The plasmid integrates into the *F. johnsoniae* chromosome. Therefore, CYE plates with 2% sucrose are subsequently used to select for *F. johnsoniae* cells that excised the plasmid from the chromosome again. The excision process has a 50/50 chance of leaving the desired modification. PCR and sequencing are used to screen colonies from the sucrose plates to identify the ones with the desired chromosomal modification.

2.3 Protein analysis

2.3.1 Sodium dodecyl sulfate and polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used routinely to determine the molecular weight, post-translational modification, purity, and

abundance of protein samples. Samples for SDS-PAGE analysis were mixed with the appropriate sample buffer and separated on 4–20% Mini-PROTEAN® TGX™ Precast polyacrylamide gels (BioRad) in SDS-PAGE running buffer (Table 2.6). To analyse very large proteins such as SprB (> 600 kDa), the Tris-Acetate NuPage™ (ThermoFisher) system was used. Samples were mixed with sample buffer to obtain a final concentration of 1x NuPAGE® LDS Sample Buffer and loaded onto 3–8% Tris-Acetate gradient gels. The gels were run with 1x NuPAGE® Tris-Acetate Running Buffer (Table 2.6). After electrophoresis, the content of the gels were either visualised by staining with Coomassie blue and destaining with destaining solution (Table 2.6) or transferred onto methanol-activated polyvinylidene fluoride (PVDF) membranes (see 2.3.2) for immunoblotting.

Table 2.6: Buffers used for SDS-PAGE. SDS=Sodium Dodecyl Sulphate. DTT=Dithioerythritol.

1x SDS-PAGE running buffer	
Tris	25 mM
Glycine	192 mM
SDS	0.1% w/v
DTT	10 mM
6x SDS-PAGE sample buffer	
Tris-HCl pH 6.8	375 mM
SDS	10% w/v
Glycerol	50% v/v
Bromophenol blue	0.1% w/v

20x NuPAGE® Tris-Acetate Running Buffer pH 8.24

Tricine	50 mM
Tris Base	50 mM
SDS	0.1% w/v

4x NuPAGE® LDS Sample Buffer pH 8.5

Tris HCl	106 mM
Tris Base	141 mM
LDS	2% w/v
Glycerol	10% w/v
EDTA	0.51 mM
SERVA Blue G250	0.22 mM
Phenol Red	0.175 mM

Coomassie blue stain

Coomassie Brilliant Blue R250	0.1% w/v
Methanol	50% v/v
Glacial acetic acid	10% v/v

Destaining solution

Methanol	15% v/v
Glacial acetic acid	10% w/v

2.3.2 Immunoblotting

Immunoblotting was used to verify the presence of proteins of interest within complex protein samples. When blotting *F. johnsoniae* total cell samples, the strains were first grown overnight in a 5 mL CYE culture. This culture was used to seed a 5 mL motility medium (MM) culture to $OD_{600}=0.05$ that was then grown for 4 hours to $OD_{600}\sim 0.5$. Cell samples corresponding to 1 mL $OD_{600}=1$ were collected and the supernatant removed (or saved in cases where the supernatant also was examined). The cells were resuspended in 100 μ L 1x SDS-PAGE sample buffer and boiled for 10 min before either proceeding further immediately or freezing for later use. Denatured protein samples in SDS-PAGE sample buffer, with dithiothreitol (DTT), unless specified otherwise, were first subjected to SDS-PAGE as described in 2.3.1. The SDS-PAGE gel was then transferred to methanol-activated PVDF membranes by using either the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad) or wet transfer in 20 mM Tris, 192 mM glycine, 10% methanol and 0.05% Tween-20 using the TE 22 Mini Tank Transfer Unit (GE Healthcare). Wet transfer of large proteins such as SprB and SprD was performed overnight at 4 °C and 20 V. Following the transfer, the transferred proteins were routinely visualised with Ponceau red (0.1 % (w/v) Ponceau S, 5% (v/v) acetic acid) and destained with water. The membranes were then blocked with 5% milk powder dissolved in TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.02% Tween-20) for 1 hour at room temperature under constant agitation.

Table 2.7: Antibodies used in this thesis.

Primary Antibody	Fold dilution	Manufacturer
Mouse α HaloTag	2000	G921A, Promega
Mouse α 6xHis tag	1000	MA1-21315, Invitrogen
Mouse α GldL	1000	(Hennell James et al., 2021)
Secondary Antibody		
Goat α Mouse	5000	A4416, Merck

The membranes were then incubated overnight at 4 °C under constant agitation with the primary antibody in 5% milk powder TBST solution at the concentration specified in table 2.7. The next day, the membranes were moved to RT and washed three times with TBST for 5 min. Subsequently, the membranes were incubated for 1 hour with secondary antibody (Table 2.7) horseradish peroxidase conjugate in 5% milk powder TBST solution under constant agitation and washed three times in TBST for 5 min per wash. Immobilon Forte HRP substrate (WBLUF0500, Merck) was applied to the membranes, and they were developed by exposing Amersham Hyperfilm ECL (GE) for 1-180 s and processed using an X-ray film processor (Optimax). Alternatively, the membranes were developed using the same substrate but imaged using a G:Box running GeneSys image acquisition software (Syngene).

2.3.3 Protease digestion of cell surface proteins

F. johnsoniae cells producing HaloTag fusion proteins were grown for 4 hours in MM and spun down at 3000 g at RT for 4 min. The supernatant was discarded and the pellet resuspended in PK buffer (Table 2.8) to an $OD_{600} = 10$. 1 mL of the resuspended solution was aliquoted into each of the 3 tubes. Cells were mixed with either 5 μ L 100mg/mL Proteinase K (Sample), 5 μ L PK buffer (Control), or 5 μ L 100mg/mL Proteinase K + 15 μ L 25% w/v Triton X-100 (Triton). Reactions were quenched at the desired time point by the addition of 10 mM phenylmethylsulfonyl fluoride (PMSF) and boiling for 3 min followed by the addition of 12 μ L 6xSDS-buffer and boiling for a further 9 min. The samples were run on 4-20% PAGE gels as described in 2.3.1 and transferred to PVDF membranes as described in 2.3.2. The membranes were first blotted with α -HaloTag antibody and thereafter with α -GldL, as described in 2.3.2. The membranes were washed for a minimum of 2 hours in TBST between the first and second blot.

Table 2.8: PK buffer pH 7.4 components.

Component	Concentration
MgCl ₂	10 mM
NaCl	136.892 mM
KCl	2.68 mM
Na ₂ HPO ₄	10.14 mM
KH ₂ PO ₄	1.76 mM

2.3.4 Assessment of colony proteolysis activity using milk plates

F. johnsoniae mutant strains were grown overnight in CYE and used to seed 5 mL MM cultures to a starting OD₆₀₀ of 0.05. The MM culture was grown for 4 hours and 2 μ L were spotted on milk-MYA agar plates. Milk-MYA agar plates were made by covering MYA agar plates with a 1 mL layer of 30% skimmed milk suspension and dried until the suspension was semi-solid. The plates were incubated overnight at 25 °C and imaged after 24 and 48 hr.

2.3.5 Crosslinking mass spectrometry

Sample treatment

Strains for analysis were grown aerobically at 30 °C in 0.5 L MM to OD₆₀₀=0.5. Cells were harvested by centrifugation at 10,000 g, 20 °C, for 20 min. The harvested cell pellet was washed twice in 350 mL phosphate-buffered saline (PBS) using the same centrifugation conditions as for the initial harvest. The washed cell pellet was resuspended in 100 mL PBS and incubated with either 0.25 mL dimethyl sulfoxide (DMSO) or 0.25 mL 100 mM DSS (21655, Thermo Scientific™) solubilised in DMSO for 45 s. The cross-linking reaction was quenched with 1mL 2M Tris pH 7.5. Cells were then spun down again at 10,000 g, 20 °C, for 20 min and resuspended in 4.5 mL Lysis buffer (Table 2.9). The cell suspension was lysed while being kept on ice using a 130 W Vibra-Cell sonicator (10 s on, 10 s off, 60% Amplitude, 15 cycles). After lysis, n-Dodecyl β -D-maltoside (D310, Anatrace)

(DDM) was added to a final concentration of 1% (w/v) and the sample was incubated for 45 min at 4 °C under constant agitation. Following solubilisation, the sample was moved to RT and mixed with urea buffer (Table 2.9). The samples were incubated overnight at room temperature. 0.5 mL agarose slurry (UltraPure™ Agarose, Invitrogen) equilibrated in urea buffer was added to the sample and incubated for 1 hour under constant agitation at RT. The agarose particles were removed by filtration through a gravity column. 100 μ L Ni-NTA agarose beads (Ni-NTA Agarose, Qiagen) equilibrated in urea buffer were added to the cleared sample and incubated under constant agitation for 2 h at RT before transferring the sample to an empty gravity column. The sample was washed three times using 25 mL urea wash buffer (Table 2.9) supplemented successively with 20, 30, and 50 mM imidazole. Finally, the proteins were eluted with urea elution buffer (Table 2.9).

Table 2.9: Buffers used for SDS-PAGE.

Components	Concentration
Lysis buffer	
Tris-HCl pH 7.5	50 mM
NaCl	150 mM
Lysozyme	30 mg
Deoxyribonuclease I bovine (D5319, Merck (Sigma-Aldrich))	20 mg
cOmplete™, Mini Protease Inhibitor Cocktail (11836153001, Merck (Roche))	1 tablet
Urea buffer	

Tris-HCl pH 7.5	50 mM
NaCl	150 mM
Urea	8M
n-Dodecyl β -D-maltoside (D310, Anatrace)	1% w/v

Urea wash buffer

Tris-HCl pH 7.5	50 mM
NaCl	500 mM
Urea	8M
n-Dodecyl β -D-maltoside (D310, Anatrace)	0.03% w/v

Urea elution buffer

Tris-HCl pH 7.5	50 mM
NaCl	500 mM
Urea	8M
n-Dodecyl β -D-maltoside	0.03% w/v
Imidazole	500 mM

The mass spectrometry preparation and execution described below were carried out by Svenja Hester at the Advanced Proteomics Facility, University of Oxford.

The elution was digested with trypsin using Filter Aided Sample Preparation (FASP) as follows (Wisniewski, 2016): The FASP filter was washed with 200 μ L 0.1 % Trifluoroacetic

acid (TFA) in 50 % acetonitrile (ACN) by centrifuging at 14,300 g for 10 min. The samples were loaded onto the filter and the proteins were denatured with 200 μ L 8M urea in 100mM Tetraethylammonium bromide (TEAB) for 30min at RT. Subsequently, the samples were reduced by adding tris(2-carboxyethyl)phosphine to a final concentration of 10 mM and incubating for 30min at RT. Samples were alkylated by adding chloroacetamide to a final concentration of 50 mM and incubating for 30 min at RT in the dark. The liquid was then removed from the filter by centrifugation at 14,300g for 10min.

To prepare for trypsin digestion the FASP filter was washed twice with 200 μ L 50mM TEAB by centrifuging at 14,300 g for 10 min each wash. Prior to adding protease, the filter was moved to a new tube.

1 μ g trypsin was added to 200 μ L 50 mM TEAB and incubated o/n at 37 °C. Following trypsin digestion, the liquid was spun through the filter and the flowthrough was saved. The filter was then washed with 200 μ L 0.1 % TFA and 200 μ L 50 % ACN in 0.1 % TFA, and the flow-through from both washes was combined. The final sample was dried in a speedVac vacuum concentrator and resuspended in 50 μ L 5% formic acid and 5% DMSO prior to running on an LC-MS/MS QExactive mass spectrometer.

Analysis of crosslinking mass spectrometry data

The mass spectra were searched against the proteome of *F. johnsoniae* UW101. Peptides and proteins were inferred using MaxQuant with a standard 1 % False Discovery Rate (FDR) at both peptide and protein assignment levels. The assigned protein groups were used for downstream analysis. The analysis of proteins identified with MaxQuant was

performed in the programming language R (Appendix 7.4). The datasets were filtered to remove proteins which were not present across all control or crosslinked datasets. In cases where protein intensity values were still 0, the value was adjusted to the minimum intensity value within the dataset. The datasets were then Log_2 transformed and normalised using the limma package (Smyth, Speed, 2003). The significance of the mean was calculated using a Welsch T-test, and the normalised log_2 -transformed intensities were plotted in a volcano plot, showing the relative fold-change of each protein in the cross-linked vs control sample on the x-axis and the significance of their mean on the y-axis. Proteins plotted in the volcano plot were coloured based on their pSORTb predicted sub-cellular localisation (Yu et al., 2010). A custom annotation of proteins of interest was amended, in cases where the pSORTb prediction was not known or incorrect according to the literature.

2.4 Microscopy data collection

2.4.1 Growth of strains for microscopy and PY2 agar plate imaging

To perform microscopy experiments with fluorophore-labelled strains expressing HaloTag or SNAP-tag fusion proteins, the following protocol for preparation of the cells was used. An ON culture grown in CYE was used to seed a 5mL Motility Medium (MM) culture to a starting $\text{OD}_{600}=0.05$. The MM culture was grown for 3 hours at 30 °C with 180 rpm shaking. Then, 1mL of the MM culture was mixed with 0.5-1 μL of a HaloTag or

SNAP-tag ligand-conjugated fluorophore dissolved in dimethyl sulfoxide (DMSO) at an appropriate concentration (Specified in the results chapters for each experiment, usually 0.01-1 nM to obtain single-molecule labelling and 100 nM to obtain saturating labelling conditions). The labelling mix was incubated for a further 30 min at 30 °C with 180 rpm shaking. Following labelling, the strains were washed 3 times with 600 μ L PY2. To ensure efficient removal of unbound dye that could stick to the side of the tube, fresh tubes were used between each wash. Following washing, the cells were suspended in 100-200 μ L PY2. In single-particle tracking (SPT) and flowcell gliding motility experiments, 50 ng/mL SynaptoGreenTM C4 dye was added to the suspension before proceeding. The suspension was then used to prepare 1 or more of the 3 types of slides: agar pads, tunnel slides, or flowcells. For PY2+1% agarose pads, 1-2 μ L of the cell suspension were deposited on top of the agarose pad. For tunnel slides or Ibidi sticky-Slide VI 0.4 flowcells, $\sim$ 40 μ L of the suspension were injected, incubated for 5 min and washed twice with 100 μ L PY2.

Agar pads were prepared as follows: PY2 medium with 1% agarose was heated in a microwave until the suspension was clear. A droplet was deposited on a 20x20 mm borosilicate coverslip (11961988, Menzel-Glaser) and incubated for $\sim$ 10 s before pressing a rectangular borosilicate coverslip 24x50 mm (11961988, Menzel-Glaser) over it, restricted by the height of two normal microscopy coverslips at either end of the rectangular coverslip. This produced a bridge that ensured the agar was not pressed completely flat while still obtaining a flat and level agar surface. The rectangular coverslip was then removed before depositing cells on the agar pad and replaced with a clean coverslip of similar brand and dimension. The glass slides were heat treated with a Furnace (unknown model,

Oxford Physics Laboratories) was necessary to remove the fluorescent background from the coverslip.

2.4.2 Imaging conditions

Imaging data were collected using highly inclined (51°) and laminated optical sheet microscopy with (SHILO) or without (HILO) strobing. The data were collected on a Nanoimager (Oxford Nanoimaging) microscope equipped with 405 nm, 532 nm, and 640 nm 1W diode-pumped solid-state lasers, 100x oil-immersion objective (Olympus, numerical aperture 1.4), and an ORCA-Flash4.0 V3 CMOS camera (Hamamatsu).

Unless otherwise stated, a 20 ms framerate was used. For single-particle tracking (SPT) experiments, SHILO movies were collected using 18% 640 nm laser power. Most often, the lasers were strobed and the strobing frequency is stated for each experiment. When collecting data using the photoactivatable dye, PAJF646-Halo, 10% 405 nm laser power was used in one of the 'off' strobing steps to avoid potential noise.

After collecting the SPT movie, in experiments where the SynaptoGreenTM C4 dye was used a 100-frame HILO movie using 25% 532 nm laser power was collected. Tracking in 3 dimensions was achieved by using an astigmatic lens that distorted the PSF as a function of the position in Z. To calculate the reference for this Z-determination, 0.1 μm tetraspec beads (T7279, Invitrogen) immobilised on a glass slide with poly-lysine were used. The slide with beads was used to collect a Z-stack ranging from -1 μm to +1 μm from focus, taking an image every 2 nm, effectively collecting a total of 1000 frames.

For flowcell gliding experiments, a constant flow of 20 $\frac{\mu\text{l}}{\text{s}}$ was used to supply fresh PY2

medium supplemented with SynaptoGreen™ C4 dye. The flow was regulated using an OB1 MK3+ microfluidic flow controller (Elveflow) equipped with an MFS-D-4 flow-rate sensor (ElveFlow) and an 8 bar 230V/50HZ 4 L compressor (Jun-Air). To capture gliding cells, SHILO movies were collected using 1 on/7 off or 1 on/24 off frequencies. Each frame that was collected was illuminated with both 12% 532 nm and 18 % 640 nm laser power. For the attachment assay (described in 2.6), 100-frame movies were collected using HILO microscopy. Each frame was illuminated with 20% 542 nm and 640 nm laser.

2.5 Image analysis

2.5.1 single-particle tracking

Microscopy data was collected as described in 2.4.2 and analysed to extract single-particle tracks (described below). In order to identify those particles that moved on helical trajectories along the cell body, the tracks were classified using a machine learning algorithm. The overall software approach from raw image data to classified tracks was as follows (Figure 2.2):

- Identify the localisations in 3D using astigmatic PSF fitting with thunderSTORM (Ovesný et al., 2014). Performed in ImageJ.
- Track and filter the localisations to identify protein movement through time and remove noise (Allan et al., 2021). Performed in the Python language.
- Classify the tracks as helical or not helical based on the movement characteristics.

Performed in the Matlab language.

- Visualise the classification distribution across multiple datasets. Performed in the R language.

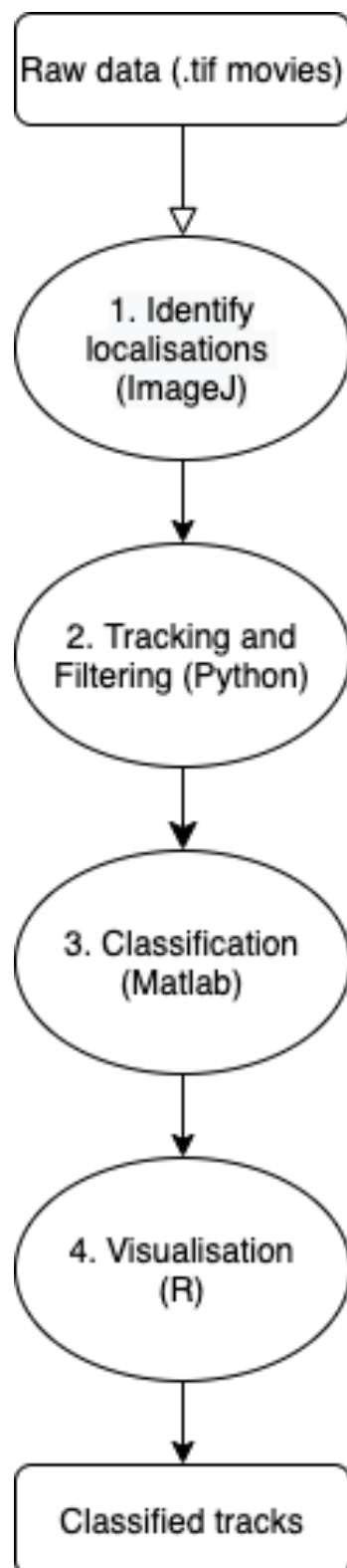


Figure 2.2: Flowdiagram describing the data flow and the high level components used during processing, including what programming language they run in.

1. and 2. Identification of fluorescent foci

Prior to the identification of molecules in the imaging data, the images were treated with outlier detection to remove 'hot' pixels with extreme intensity values. For this, the built-in ImageJ program 'Remove Outliers' was used with the settings described in Table 2.10:

Table 2.10: ImageJ 'Remove Outliers' settings used. Block radius is given in pixels. Standard deviations refer to pixel intensity.

Remove Outliers	
Block radius X	40
Block radius Y	40
Standard deviations	25

Following the removal of outliers, molecules were localised with the ImageJ plugin thunderSTORM using the settings given in Table 2.11 (Ovesný et al., 2014). The reference stack collected on tetraspec beads as described in 2.4.2 was used to generate the calibration file used to determine the Z-position of each localisation in 3D with thunderSTORM.

Table 2.11: thunderSTORM settings used to process imaging data.

Camera Settings	
Pixel Size [nm]	116.9

Photoelectrons per A/D count	0.46
Base level [A/D counts]	300
EM Gain	FALSE

Image filtering

Filter	Waveline Filter (B-Spline)
B-Spline order	3
B-Spline scale	2

Approximate localization of molecules

Method	Local maximum
Peak intensity threshold	1.2*std(Wave.F1)
Connectivity	8-neighbourhood

Sub-pixel localisation of molecules

Method	PSF: Elliptical Gaussian (3D astigmatism)
Fitting radius [px]	6
Fitting method	Maximum likelihood
Initial sigma [px]	1.6
Multi-emitter fitting analysis	FALSE
Calibration file	PATH/TO/YOUR/CALIBRATION/FILE

Visualisation of the results

Method	Normalized Gaussian
Magnification	5
Update frequency [frames]	50
3D	TRUE
Colorize z-stack	TRUE
Z range (from:step:to) [nm]	-500:10:500
Lateral uncertainty [nm]	20, Force=FALSE
Axial uncertainty [nm]	100, Force=FALSE

Localisations identified with thunderSTORM were tracked using a custom python script that implemented the *trackpy* package (Allan et al., 2021). The architecture of the script is shown in Figure 2.3 and consists of 3 steps: a,b, and c (Figure 2.3).

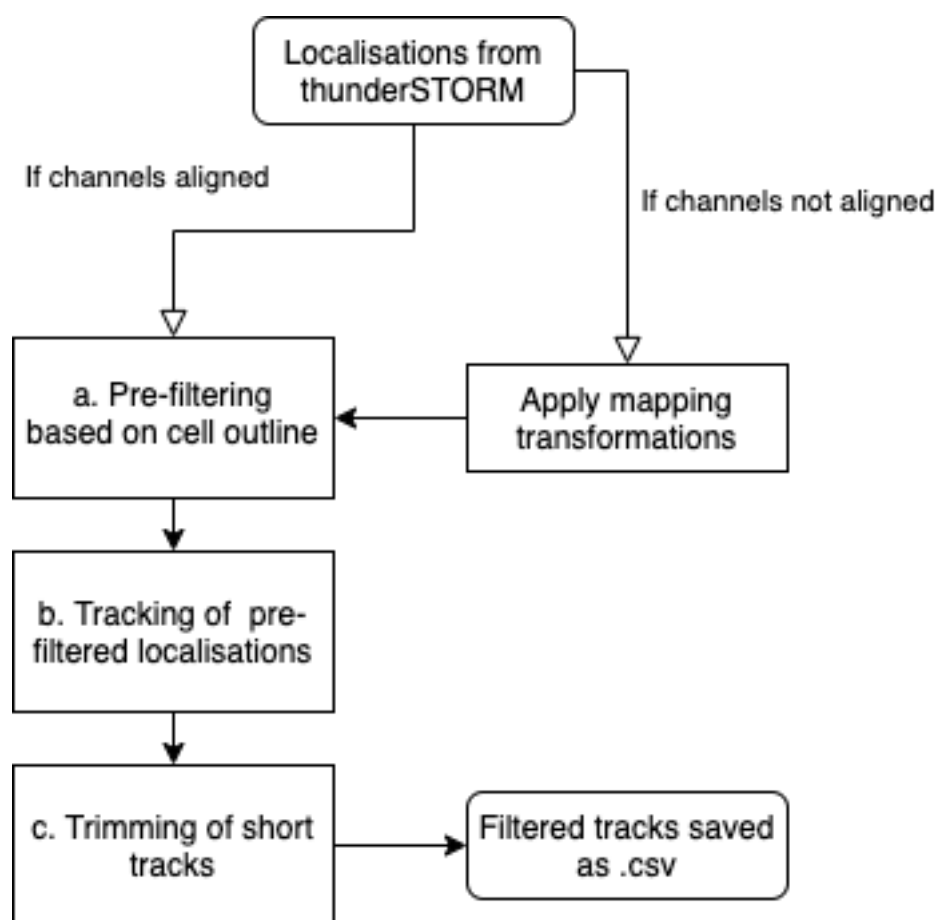


Figure 2.3: Flowchart representing the different steps of the custom python script used to perform tracking and filtering of localisations.

a. Pre-filtering based on cell outline

To remove localisations that were positioned in non-cell areas of the images, I used the data from the 532 nm excited channel corresponding to the signal from the cell membrane dye signal. Cell segmentation was used to restrict localisations, to those that were within a cell boundary. Segmentation was done by canny edge detection (Canny, 1986) using fixed parameters of lower = 1.2 and upper = 5 thresholds (Figure 2.4). Following canny

edge detection, the stack of edge images was averaged and the resulting single image was thresholded using Otsu’s method to obtain a binary image (Otsu, 1979). The binary image was then dilated to bridge edges of cells that were not complete/closed (Figure 2.4). Subsequently, the binary cell outlines were filled and a series of erosion-dilation procedures were used to remove/minimise noise. The resulting binary image was used to find contours (i.e. outlines of each blob, Figure 2.4). Fluorescent dirt in a microscope slide appeared as a circular shape unless the fluorescent object was larger than the point spread function and non-circular. Most commonly this was not the case and thus the majority of noise was readily removed by filtering away circular objects (Figure 2.4). Circular objects were removed by filtering each object based on the area and aspect ratio of a fitted ellipse. Ellipse-shaped objects that passed the aspect ratio and area test had their convex hull calculated. Because edge detection sometimes failed to segment the cells completely, the convex hull rescued some of these failed cells. The resulting shapes were redrawn onto a black image thus producing a filtered binary image (Figure 2.4). This final binary image was then used as a filter to subset the localisations with (Figure 2.4).

The tracking algorithm links a given localisation in frame i to the closest localisation in the following frame (i.e. $i+1$). Therefore, the tracking quality depends on the proximity of localisations to each other. A common source of tracking errors is when two molecules pass by each other. To avoid this, and minimise data loss, a moving window covering 10 frames was used to drop localisations where 2 frames out of the 10-frame window contained 2 or more localisations. This filtering procedure was performed for each cell area that was identified and produced an acceptable trade-off between improved tracking

and data loss, as removing all duplicate molecules resulted in too many gaps that broke longer tracks apart.

b. Tracking of pre-filtered localisations

The resulting set of filtered localisations was then linked together in time. This was done by using a dedicated tracking package: *trackpy* (Allan et al., 2021). An adaptive search algorithm which alters the search distance in an iterative process to find the most likely localisation in the next frame was used.

c. Trimming of short tracks

In order to identify helical tracks, the duration of the tracks must be long enough to produce a recognisable helical shape. Therefore, tracks shorter than 50 frames were removed.

This was done using the *trackpy* package function: *filter_stubs*.

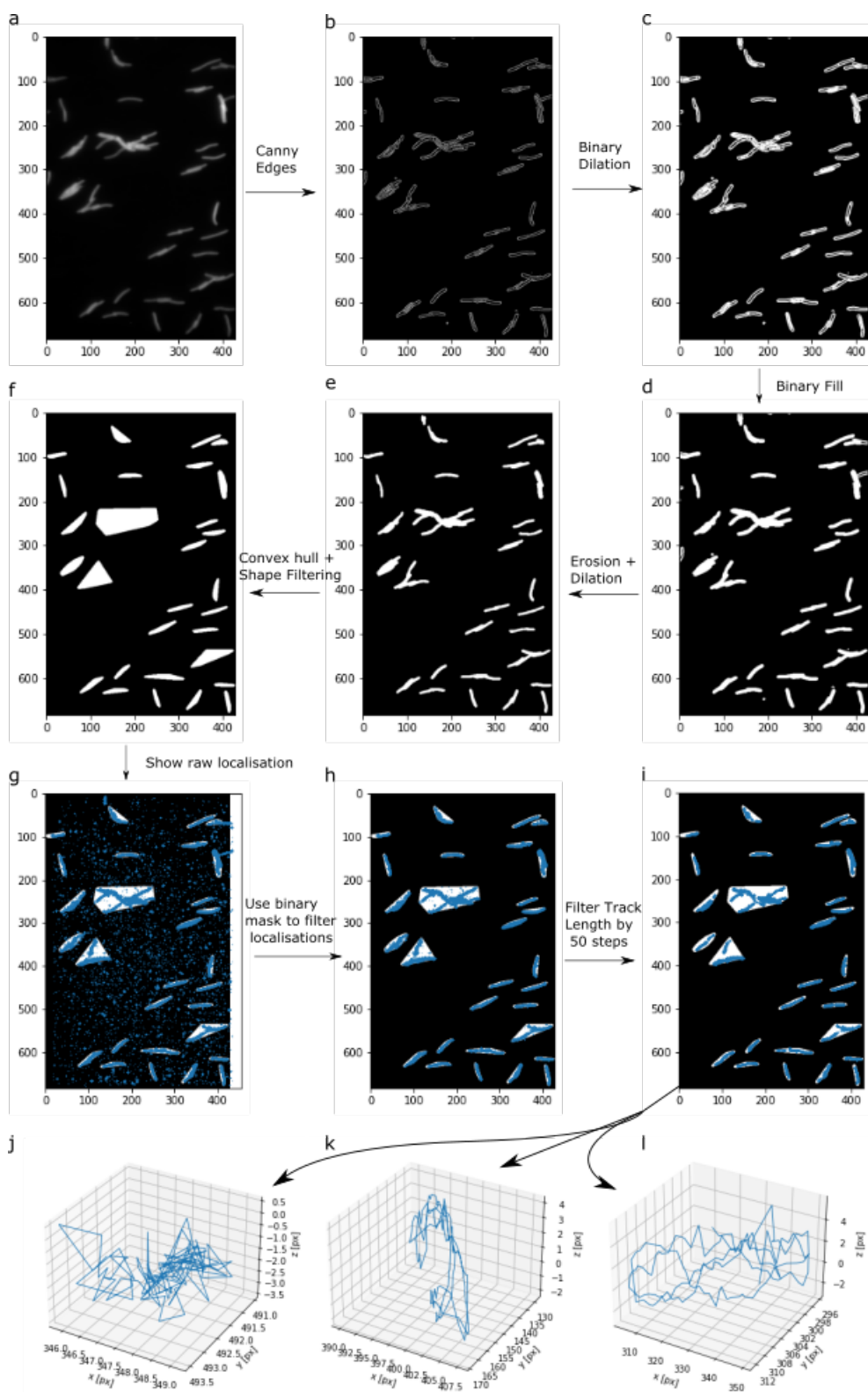


Figure 2.4: Example images of the cell segmentation and localisation filtering. The cell membrane signal (**a**) is used as input. The Canny Edge filter is applied to find edges in the image (**b**). These edges are dilated (**c**) to ensure the cell shapes are closed before filling closed shapes (**d**). To remove small noise objects, erosion followed by dilation is performed (**e**). Sometimes the program fails to find the whole cell and therefore a greedy optional choice is implemented, allowing the user to compute the convex hull of each object, thus increasing the binary area. By filtering the shapes by their circularity and aspect ratio, only ellipsoid shapes are retained (**f**). The resulting binary mask is used to remove all localisations outside the cell shapes (**g-h**) and a subsequent minimum track length ensures that short tracks are removed (**i**). Examples of 3D tracks that are Brownian (**j**) and helical (**k-l**) are shown.

3. Classification

The coordinates of the filtered tracks were loaded into a custom Matlab script that classified each track as helical or not, based on the type of movement. An overview of the process can be seen in figure 2.5

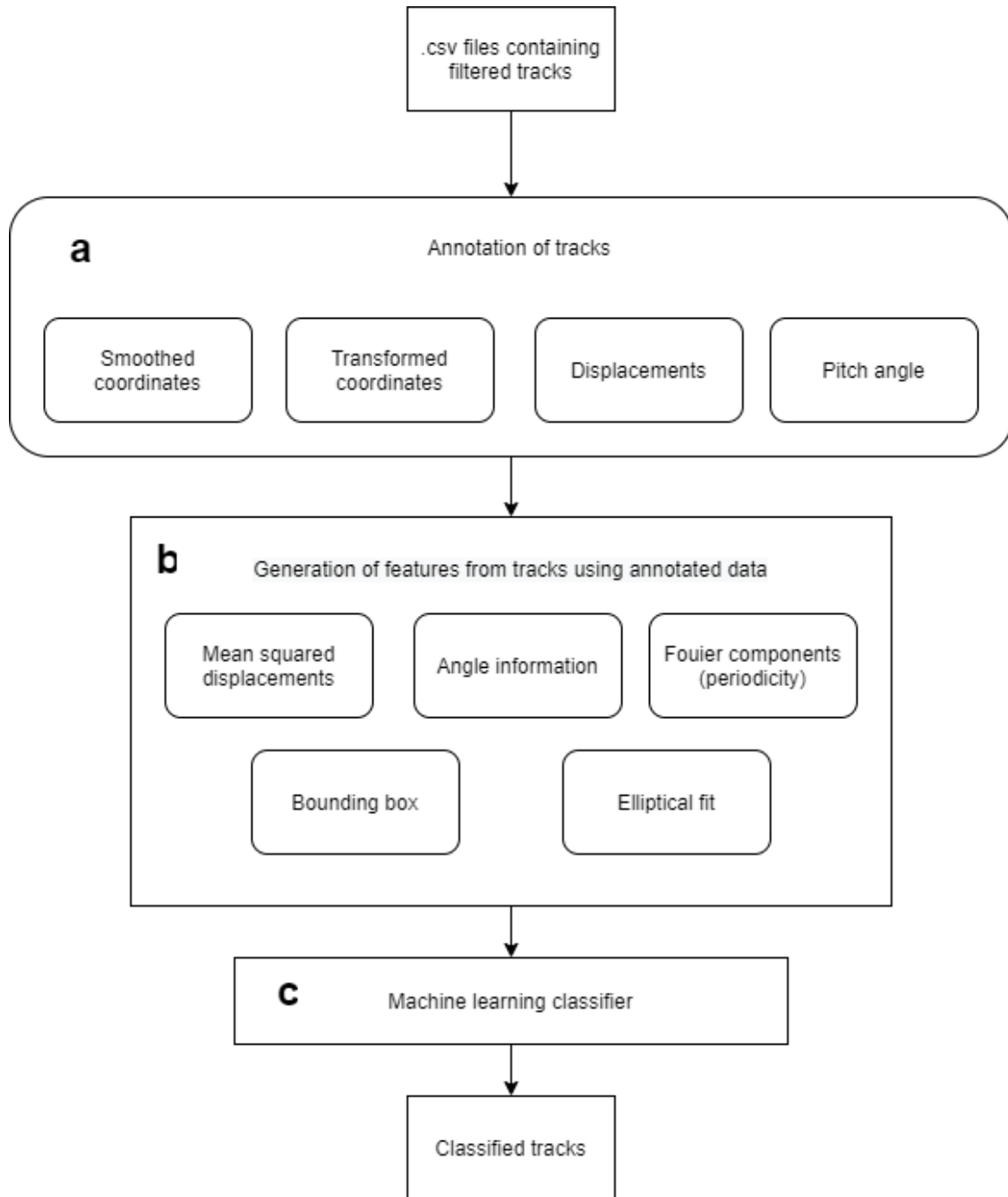


Figure 2.5: The tracks, stored in .csv files, are loaded into the Matlab program. **(a)** Using the coordinates of the track, smoothed and transformed coordinates, displacements, and pitch angles are calculated. **(b)** Single value information is generated from the data in **a** as well as additional data such as the bounding box and parameters of an elliptical fit. **(c)** The data is passed to a random forest machine learning classifier that labels each track as either helical or not helical.

a. Annotation of tracks

Missing data points in the track were filled by linear interpolation. The track data was then annotated with derivations of the data points that were used to mine features from in the later steps. The data are listed in table 2.12. Transformation of the (X, Y) coordinates to align the track along its long axis and centre it around 0,0 (X, Y) were computed by defining the long axis as the axis between the two most extreme points. The second axis was then defined as the distance of each point to this long axis. The Z-axis was already roughly centred in 0 and so did not require coordinate transformation. The consecutive coordinates were smoothed through time in each dimension (X, Y, Z) using a 5-frame window. Displacements in each dimension were calculated by measuring the successive distance between points through time (Equation 2.1).

$$1D \text{ displacement} = \begin{bmatrix} \sqrt{(x_2 - x_1)^2} \\ \sqrt{(x_3 - x_2)^2} \\ \vdots \\ \sqrt{(x_{i-} - x_{i-2})^2} \\ \sqrt{(x_i - x_{i-1})^2} \end{bmatrix} \quad (2.1)$$

The 3-dimensional helix pitch angle (θ) was calculated for the transformed coordinates, point-to-point, using Equation 2.2 with a 5 frame distance.

$$\cos\theta = \frac{\vec{dr1} \cdot \vec{dr2}}{\|\vec{dr1}\| \|\vec{dr2}\|} \quad (2.2)$$

In Equation 2.2 $d\vec{r}1$ refers to the point-to-point vector of the data (Equation 2.3) and $d\vec{r}2$ is the point-to-point change in the x-axis coordinate with Y and Z set to zero (Equation 2.4).

$$d\vec{r}1 = \begin{bmatrix} x_5 - x_0 & y_5 - y_0 & z_5 - z_0 \\ x_6 - x_1 & y_6 - y_1 & z_6 - z_1 \\ \vdots & \vdots & \vdots \\ x_{i-1+5} - x_i & y_{i-1} - y_i & z_{i-1} - z_i \\ x_{i+5} - x_i & y_{i+5} - y_i & z_{i+5} - z_i \end{bmatrix} \quad (2.3)$$

$$d\vec{r}2 = \begin{bmatrix} x_5 - x_0 & 0 & 0 \\ x_6 - x_1 & 0 & 0 \\ \vdots & \vdots & \vdots \\ x_{i-1+5} - x_i & 0 & 0 \\ x_{i+5} - x_i & 0 & 0 \end{bmatrix} \quad (2.4)$$

Table 2.12: Columns in a typical track .csv file.

Columns of data before annotation	
1	Channel
2	cur_frame
3	Track_ID
4	X_position

5	Y_position
6	Z_position
7	Photon_count
8	Diffus_Coeff

Data generated from the above input (Annotations)

9	Smoothed X (=Xs)
10	Smoothed Y (=Ys)
11	Smoothed Z (=Zs)
12	centered/rotated Xs
13	centered/rotated Ys
14	centered/rotated X
15	centered/rotated Y
16	X 1D displacement
17	Y 1D displacement
18	Z 1D displacement
19	Smoothed X (Xs) 1D displacement
20	Smoothed Y (Ys) 1D displacement
21	Smoothed Z (Zs) 1D displacement
22	angle3D computed with a window

b Generation of ML features

The annotated data was then passed to the ML Classification function which summarised

the calculated data into single-value entities that could be used as input for a random forest ensemble classification algorithm. There were multiple summations performed which included the mean squared displacements (MSD) of all combinations of 1, 2, and 3 dimensions, the 3 largest wave components extracted via Fourier transformation from both the coordinates and 3D-angles, parameters of an ellipse fit to the shape of the track as well as the dimensions of the bounding box that encompasses the track.

The MSD was calculated for each track by splitting the track in a $30(n)$ -frame window starting from each frame index, j (Equation 3.6). The distance of a starting point (Equation 3.7 $\vec{A}$) to all points from x_j to x_{j+n} (Equation 3.7 $\vec{B}$) was calculated (Equation 3.8 $\vec{D}$) and differentiated (Equation 3.8 $\vec{D}'$) to obtain the MSD at all lag-times and the slope of that line, respectively. The average slope was used as the MSD for each j and stored in a new vector (Equation 3.9 MSD).

$$n = 30, j = 1, 2 \dots \#frames \text{ in track} - n \quad (2.5)$$

$$\vec{A} = n \text{ elements} \left\{ \begin{bmatrix} x_j \\ x_j \\ \vdots \\ x_j \end{bmatrix} \right\}, \vec{B} = \begin{bmatrix} x_j \\ x_{j+1} \\ \vdots \\ x_{j+n} \end{bmatrix} \quad (2.6)$$

$$\vec{D} = \sqrt{A^2 - B^2}, \vec{D}' = \begin{bmatrix} D_2 - D_1 \\ D_3 - D_2 \\ \vdots \\ D_n - D_{n-1} \end{bmatrix} \quad (2.7)$$

$$MSD(j) = \frac{1}{n} \sum_{i=1}^n D'_i = \frac{D'_1 + D'_2 + \dots + D'_n}{n} \quad (2.8)$$

The MSD calculation was extended to encompass d dimensions by modifying $\vec{A}$, $\vec{B}$, and $\vec{D}$ (Equation 3.10 and 3.11).

$$d = 3, \vec{A} = n \text{ elements} \left\{ \begin{bmatrix} x_j & y_j & z_j \\ x_j & y_j & z_j \\ \vdots & \vdots & \vdots \\ x_j & y_j & z_j \end{bmatrix}, \vec{B} = \begin{bmatrix} x_j & y_j & z_j \\ x_{j+1} & y_{j+1} & z_{j+1} \\ \vdots & \vdots & \vdots \\ x_{j+n} & y_{j+n} & z_{j+n} \end{bmatrix} \right. \quad (2.9)$$

$$\vec{D} = \sqrt{\sum_{i=1}^d A_{*,i}^{\vec{2}} - B_{*,i}^{\vec{2}}} = \sqrt{(A_{*,1}^{\vec{2}} - B_{*,1}^{\vec{2}}) + (A_{*,2}^{\vec{2}} - B_{*,2}^{\vec{2}}) + (A_{*,3}^{\vec{2}} - B_{*,3}^{\vec{2}})} \quad (2.10)$$

The data were fitted to the shape of an ellipse (Equation 3.12). The shape was fitted to the transformed coordinates (centred in 0) using a least-squares estimation method without weights written in the Matlab language and available online (Gal, 2003).

$$Ellipse = ax^2 + bxy + cy^2 + dx + ey + f = 0 \quad (2.11)$$

For the machine learning algorithm, the ellipse was represented by each of the fitting parameters that were estimated as well as the residual sum of squares from the distance between the fitted ellipse and the data points. The residual sum of squares was calculated using the publicly available *Residuals_ellipse* Matlab function (Ma, 2010). Furthermore, the minimum, maximum, mean, and variance of the distance between the fitted ellipse and the data were calculated and included as features in the ML program using built-in Matlab functions.

Bounding boxes were calculated for each 2-dimensional combination of the X, Y, and Z dimensions. The calculation was done for both the raw and smoothed coordinate set to account for any smoothing effect that would potentially diminish important differences between helical and non-helical tracks.

c Machine learning classifier

Following the preparation of the feature table, the features were used to train a random forest classification algorithm. 5392 Tracks from mutant strains with and without the ability to produce helically moving molecules were sorted manually and used to train the algorithm. The algorithm was chosen by assessing the accuracy of a range of algorithms available in the "Classification Learner" App in Matlab, using 5-fold cross-validation. The details of the chosen algorithm can be seen in Table 2.13. As part of the machine learning training, a confusion matrix is produced that summarises the right and wrong

classifications compared to the manually curated training set. This confusion matrix was used to calculate the categorical percentage errors reported in Figure 3.10.

Table 2.13: Settings used to train and validate the machine learning algorithm.

Category	Value
Algorithm details	
Preset	Optimizable Ensemble
Ensemble Method	GentleBoost
Learner type	Decision Tree
Maximum number of splits	14
Number of learners	48
learning rate	0.039272
Hyperparameter Search range	
Ensemble method	Bag, GentleBoost, Logit-Boost, AdaBoost, RUSBoost
Number of learners	10-500
learning rate	0.001-1
Maximum number of splits.	1-5391
Number of predictors to sample	1-81
Optimizer Options	
Optimizer	Bayesian optimization
Acquisition function	Expected improvement per second plus

Category	Value
Iterations	30
Training time limit	false
Validation and training	
validation technique	5-fold Cross-validation
Number of tracks in training set	5392
Accuracy	98 %

2.5.2 Statistical analysis of 3-dimensional single-particle tracking data

Tracking data obtained from the classification method described above were modelled and tested in the R programming language. A generalised additive model (GAM) implemented in the *gamlss* R package (Rigby, Stasinopoulos, 2005) was used with a zero-inflated beta distribution. To account for the varying number of tracks analysed across different days and strains, the total track count was included as a weighted argument.

The proportion of all tracks that were classified as helical for each field of view and these values were used as the response variable. The strain was used as the dependent variable. The data was presented with boxplots that were generated using the *ggplot* package in the R programming language. The box shows the inter quartile range (IQR) from the 25th to 75th percentile. The center is the 50th percentile. The upper whisker shows the highest

value that is below $1.5 * \text{IQR}$ from the 75th percentile and the lower whisker shows the lowest value above $1.5 * \text{IQR}$ from the 25th percentile. Red dots were drawn on top of the boxplot to show the average value of a biological replicates that were used to produce the boxplot.

Pairwise comparisons were done using the estimated marginal means (*emmeans*) package (Searle et al., 1980) and the *p*-values were *post hoc* corrected using the Bonferroni method (Bonferroni, 1936).

2.6 Attachment assay

To assess competitive attachment to the glass between parental and mutant strains, I prepared the strains as described in 2.4.1. The strains were then labelled with either JF646 or JF549. After labelling, OD_{600} measurements were taken to guide the amounts of each strain that needed to be mixed to achieve near 1:1 ratios of the two cell types. The strains were washed individually as described in 2.4.1 and pooled before depositing on agar pads or injecting into tunnel slides.

Following imaging, all cells were counted manually using the cell counter plugin in ImageJ. Thereafter, all data analysis was performed in the R programming language. For each field of view, the ratio of mutant to parental strain was calculated. All the resulting ratios for a given day and strain were averaged to obtain a single ratio for agar pads and tunnel slides.

The number of mutant cells was divided by the number of parental cells for both the agar

pad and tunnel slide datasets. The ratio of mutant to parental strain from tunnel slides was divided by the corresponding agar pad ratio to adjust for uneven loading. The data was modelled with a generalised least squares model implemented in the *gls* package and pairwise comparisons to either the parental or the *halo::sprB_{CTD448}* strain were specified *a priori* and tested using the *glht* package. The *p* values were corrected using the Shaffer method (Shaffer, 1995).

2.7 Bioinformatics

2.7.1 Conservation of *sprB* operon proteins

Refseq genomes from the Cytophaga, Fusobacterium, and Bacteroides (CFB) group were downloaded from the NCBI FTP server and the genomes with annotated open reading frames were selected. This yielded ~1000 genomes. Hidden Markov model (HMM) profiles for the proteins/domains of interest were downloaded from TIGRFAM (SprF/TIGR03519, type A CTD/TIGR04183, type B CTD/TIGR04131). HMM profiles were created for SprC, RemG, and RemF by searching with their protein sequence for homologs using the online HMMER web server against the Uniprot KB database restricted to the *Bacteroidetes* phylum, downloading the aligned hits, and using the alignment to create an HMM using *hmmbuild* from the hmmsuite command-line tools (Eddy, 1998). All HMM models were used to search through the genomes using the hmmsuite command *hhsearch*. By iterating through all the retrieved *sprF* genes, all genes within a 5-gene distance up- or downstream from *sprF* were retrieved. Of these neighbourhood genes, all sets that

did not have *sprB*, *remG*, and *sprC* were discarded and the remaining sets were used to draw a phylogenetic tree. The HMM profile for SprD was not specific enough to use for subsetting the genomes. The phylogenetic tree was made using the NCBI taxonomy database. A minimal tree connecting all the selected genomes was retrieved and a tree was drawn with each leaf annotated with a 50 kb genome stretch centred around *sprF* with coding sequences highlighted and coloured according to the HMM hit.

A tree was also drawn of all *Bacteroidetes* genomes with coloration indicating presence/absence of *gldJ*. This was done by searching for GldJ-encoding genes with the TIGR03524 HMM. The tree was in this case also retrieved from the NCBI taxonomy database.

2.7.2 Prediction of secondary structure

Psipred was used to predict the secondary structure of protein sequences. The program uses position-specific scoring matrices from a position-specific iterative basic local alignment search tool and feeds the input into a 2-state neural network (Jones, 1999). The output of the network is either strand, helix, or coil with an average accuracy of 75–80%.

2.7.3 Structure prediction from sequence data

For the majority of the proteins investigated in this thesis, there was no structure available. Therefore, the structure of these proteins was predicted by structural homology matching using the program.

Protein Homology/analogY Recognition Engine V 2.0 (Phyre2) (Kelley et al., 2015). Phyre2 generates a profile from a query sequence, searched against a non-redundant

database using HHblits (Remmert et al., 2012). In addition, the secondary structure is predicted using psipred (described above), and both the sequence profile and secondary structure are then converted into an HMM used to search against a protein structure database of similarly calculated HMMs. The structures matched by the HMM-HMM search are used to build a backbone model of the predicted structure. Gaps in the backbone are modelled from a library of fragmented sequences, and then the remaining backbone is built using virtual ribosome synthesis in a crowded environment (Poing) (Jefferys et al., 2010). The side-chains are built using PULCHRA (Rotkiewicz, Skolnick, 2008) and fitted using the global optimisation algorithm described in (Xie, Sahinidis, 2006).

Chapter 3

Development of an adhesin tracking assay

3.1 Introduction

F. johnsoniae is a member of the *Bacteroidetes* phylum that is capable of gliding motility.

Bacteroidetes gliding motility is the movement across surfaces by helical propulsion of adhesin molecules on the cell surface. The *F. johnsoniae* genome contains numerous adhesin genes. However, the adhesin SprB has been shown to be responsible for all colony spreading on PY2 agar and all but trace movement on glass (Nelson et al., 2008). Thus, SprB is functionally the most important adhesin.

SprB is large (6497 amino acids), consisting of ~500 amino acid (aa) repeat sequences that contain further smaller internal repeat sequences. The largest truncation of SprB

constructed so far, containing 1182 aa's from the C-terminal end of SprB, is unable to support detectable SprB-mediated cell motility (Kulkarni et al., 2019). SprB movement has been studied with SprB antibody-coated polystyrene spheres (Nelson et al., 2008) and by immunofluorescence (Nakane et al., 2013)(Kulkarni et al., 2019). To avoid the complications involved in trying to follow adhesin movements when the cell body is also moving and rolling, Nakane et al. (2013) analysed a subpopulation of cells that did not move when attached to glass whereas Kulkarni et al. (2019) assessed movement of SprB on cells in solution. In both cases, SprB were reported to move along the cell body in a helix with a $\sim 20^\circ$ pitch angle and at a constant speed of $\sim 2 \frac{\mu m}{s}$.

Bacteroidetes gliding motility requires the activity of the phylum-specific Type 9 Secretion system (T9SS) to export the gliding adhesins to the cell surface. The T9SS secretes, in a proton motive force (PMF) dependent manner, a range of substrates from the periplasmic to extracellular space. The proteins GldKLMNO and SprAET are components of the *F. johnsoniae* T9SS. GldKLMNO forms a trans-periplasmic complex that transduces the proton motive force (PMF) to power protein transport (Hennell James et al., 2021). SprA contains a large pore through which substrates are secreted across the outer membrane (Lauber et al., 2018). Substrate proteins are targeted to the T9SS by their C-terminal domain (CTD). Two classes of CTD have been identified. Type A CTDs are transferred from the SprA channel to the shuttle protein PorV. The secondary adhesin RemA is an example of a protein with a type A CTD. Other substrate proteins carry the Type B CTD. These proteins do not require PorV but instead interact with a specialised partner protein of the PorP family. The major *F. johnsoniae* adhesin, SprB, possesses a Type B

CTD and uses the PorP family partner protein, SprF, for secretion by the T9SS. Despite having other adhesins encoded in the genome, SprB has been shown to be required for colony spreading on PY2 agar plates (Nelson et al., 2008).

In addition to the T9SS, dedicated gliding motility proteins (GldA-J) are required for functional gliding in an unknown manner. It is believed that one or more of these proteins form a helical track in the periplasm and that this track provides direction and power to the helically moving adhesins (Shrivastava, Berg, 2020). So far it has been difficult to directly study any of the GldA-J proteins in great detail, and therefore little is known about how the helical organisation of the track is achieved. (Braun, McBride, 2005) were able to show by immunofluorescence microscopy that GldJ is likely organised in a helical structure suggesting that this protein might form the track. Deletion of any of the *gldA-I* genes leads to GldJ degradation, which in turn results in the degradation of the T9SS-core protein GldK (Johnston et al., 2018). This behaviour makes it challenging to study the function of the individual gliding machinery components.

Establishing the exact pattern and dynamics of the gliding tracks is crucial to understanding the basis of gliding motility. For this reason, at the start of my project, I made extensive efforts to fluorescently tag the putative track protein GldJ. However, I was unable to find a position in GldJ where I could insert a reporter protein domain without abolishing the gliding function. I, therefore, needed to find a different approach to study the organisation of the track machinery. I reasoned that the path that SprB follows indirectly reveals the location of the underlying track. Consequently, I decided to characterise the movement of SprB in detail using single-particle tracking (SPT). In comparison to the

earlier studies of SprB movement (Nakane et al., 2013)(Kulkarni et al., 2019), SPT experiments allow higher precision in the adhesin localisation and the ability to follow the path of a single adhesin molecule as it moves multiple times along the cell body. The following sections address the development of a methodology to allow SPT of SprB in 2 and 3 dimensions.

3.2 Results

3.2.1 Construction of a Halo-SprB fusion

When performing SPT, fluorescent fusion proteins are often used to visualise the protein of interest, as they are easy to incorporate into the genome of the studied organism. Brightness and photostability are key elements of the fluorescent tag. A bright fluorophore allows good estimation of the point spread function (PSF), resulting in increased localisation accuracy. Increased photostability of a fluorophore increases the time a fluorophore can be observed before it bleaches. Fluorophores that blink should be avoided because they result in reduced temporal knowledge of the moving particle.

I decided to use the HaloTag technology to label SprB. HaloTag enables synthetic dyes with high photostability and brightness to be covalently linked to a fusion protein containing the HaloTag domain. HaloTag is a self-labelling enzyme that creates an irreversible covalent bond between itself and a ligand containing a haloalkane group (England et al., 2015). JaneliaFluor-646 (JF646) and JaneliaFluor-549 (JF549) are particularly bright and stable dyes that can be used to label HaloTag (Grimm et al., 2015) and are the dyes used

in this study. HaloTag is a relatively small protein (33 kDa) which reduces the chance that it will interfere with the function of the protein to which it is fused.

I constructed strains in which HaloTag is fused to the N-terminus of either full-length SprB, the 218 or 448 C-terminal residues of SprB. These strains were constructed by appropriate alteration of the chromosomal *sprB* gene inserting both a twin-strep affinity tag and a HaloTag after the SprB signal peptide without use of any linker regions. The resulting alleles are designated, *Twin-Strep-tag::halo-SprB*, *Twin-Strep-tag::halo-SprB_{CTD218}*, and *Twin-Strep-tag::halo-SprB_{CTD448}*. I included the Twin-Strep-tag sequence because I initially intended to purify the constructs using Strep-tag affinity purification. However, I was unable to detect the Strep-tag sequence using anti-Strep antibodies, suggesting proteolysis of the tag (data not shown). For simplicity, I will omit "*Twin-Strep-tag*" when referring to the mutant and "Twin-Strep-tag" when referring to the protein product in the remainder of the thesis.

The functionality of the Halo-SprB fusions was assessed by examining their ability to support colony spreading on PY2 agar plates. Wild type (WT) and *halo::SprB* strains were both capable of spreading on PY2 agar plates, although the presence of the HaloTag on SprB did inhibit spreading slightly. The fusion proteins containing truncated SprB domains did not support spreading, confirming previous observations by (Kulkarni et al., 2019) (Figure 3.1 a). To see if I could visualise the helical motion of the SprB fusion proteins, I labelled Halo-SprB, Halo-SprB_{CTD448}, and Halo-SprB_{CTD218} producing strains with JF646-Halo, immobilised the cells in agarose, and imaged them using highly inclined and laminated optical sheet (HILO) microscopy. Despite low levels of adhesin labelling

compared to the antibody-mediated labelling study of Nakane et al. (2013), I did observe characteristic helical movement for all SprB fusion proteins (data not shown). The low levels of labelling were likely due to proteolytic cleavage of the fusion proteins as previously reported for a fusion of SprB to sfGFP by Kulkarni et al. (2019).

To understand how the proteolytic degradation of the Halo-SprB fusion proteins might be avoided the extracellular proteolytic activity of *F. johnsoniae* was investigated. *F. johnsoniae* can utilise various protein sources as sole C, N, and energy sources (Christensen, 1947) and has 124 predicted peptidases encoded in its genome (McBride et al., 2009). Some of these predicted peptidases have type A CTDs and are predicted to be secreted by the *F. johnsoniae* T9SS in a PorV-dependent manner. As SprB does not require PorV for export (above) I hypothesised that deleting *porV* might prevent the secretion of the proteases responsible for the proteolysis of the Halo-SprB fusions without affecting the correct localisation of the SprB fusion proteins. I decided to assess the effect of *porV* deletion on secreted proteolytic activity using MYA plates containing milk protein. In this assay proteolysis of the milk proteins form a clear zone around the colony. By using a *halo-SprB_{CTD448}* background for these experiments that is unable to mediate colony spreading on PY2 agar plates, I was able to study the clearing phenotype without the confounding effect of colony spreading (Figure 3.1 b). After 1 day of incubation, a clear difference in the clearing was observed between the parental and $\Delta porV$ strains, with no noticeable clearing detected around colonies of the latter strain. After 2 days, proteolytic activity was seen with $\Delta porV$ as well as $\Delta sprA$, suggesting there are also PorV- and T9SS independent proteases (Figure 3.1 b).

Since the $\Delta porV$ *halo-SprB_{CTD448}* strain had reduced proteolytic activity, I tested whether the $\Delta porV$ background reduced proteolysis of the Halo-SprB fusion (Figure 3.1 **c**). The SprB fusion protein was indeed more stable in the $\Delta porV$ background showing no proteolytic release of the HaloTag domain in the first 6 hours of growth, although the fusion proteins was degraded. A $\Delta porV$ background also blocked proteolysis of the full length Halo-SprB fusion (Figure 3.1 **d,e**).

In an attempt to identify the specific proteases that could be responsible for the cleavage of the Halo-SprB fusions, I deleted the genes for two PorV-dependent proteases Fjoh_2389 and Fjoh_0798 that were previously identified by mass spectrometry (Lauber et al., 2018). However, removing one or both of these enzymes had only a weak effect on the proteolysis of Halo-SprB indicating that additional proteases are involved in degrading the fusion protein (Figure 3.1 **d-e**). As the exact mechanism by which $\Delta porV$ positively affects Halo-SprB stability is not required for the SPT analysis of Halo-SprB movement, the identity of the proteases responsible for proteolysis of the fusion proteins was not investigated further.

In addition to proteolysis, another potential reason for the low numbers of helical moving Halo-SprB is if the fusions are not successfully targeted to the cell surface. To test whether the fusion was incorrectly localised I carried out protease protection experiments. If Halo-SprB fusion proteins were successfully secreted they should be susceptible to proteolysis by external proteinase K, whereas if there is an export defect the fusion proteins would remain inside the cell and not be digested. Cells expressing the Halo-SprB_{CTD448} fusion were treated with proteinase K. Immunoblotting showed that the fusion protein was fully

accessible to the protease and thus confirmed that it is surface exposed (Figure 3.1 **f**). Control blots showed that the cytoplasmic membrane protein GldL was protected from proteinase K in intact cells and thus that the protease cannot cross the *F. johnsoniae* outer membrane. Furthermore, the control blots showed that proteinase K was fully inactivated before the sample preparation for immunoblotting. GldL was digested when the cells were permeabilised with detergent confirming that GldL is susceptible to digestion by proteinase K (Figure 4.3 **f**).

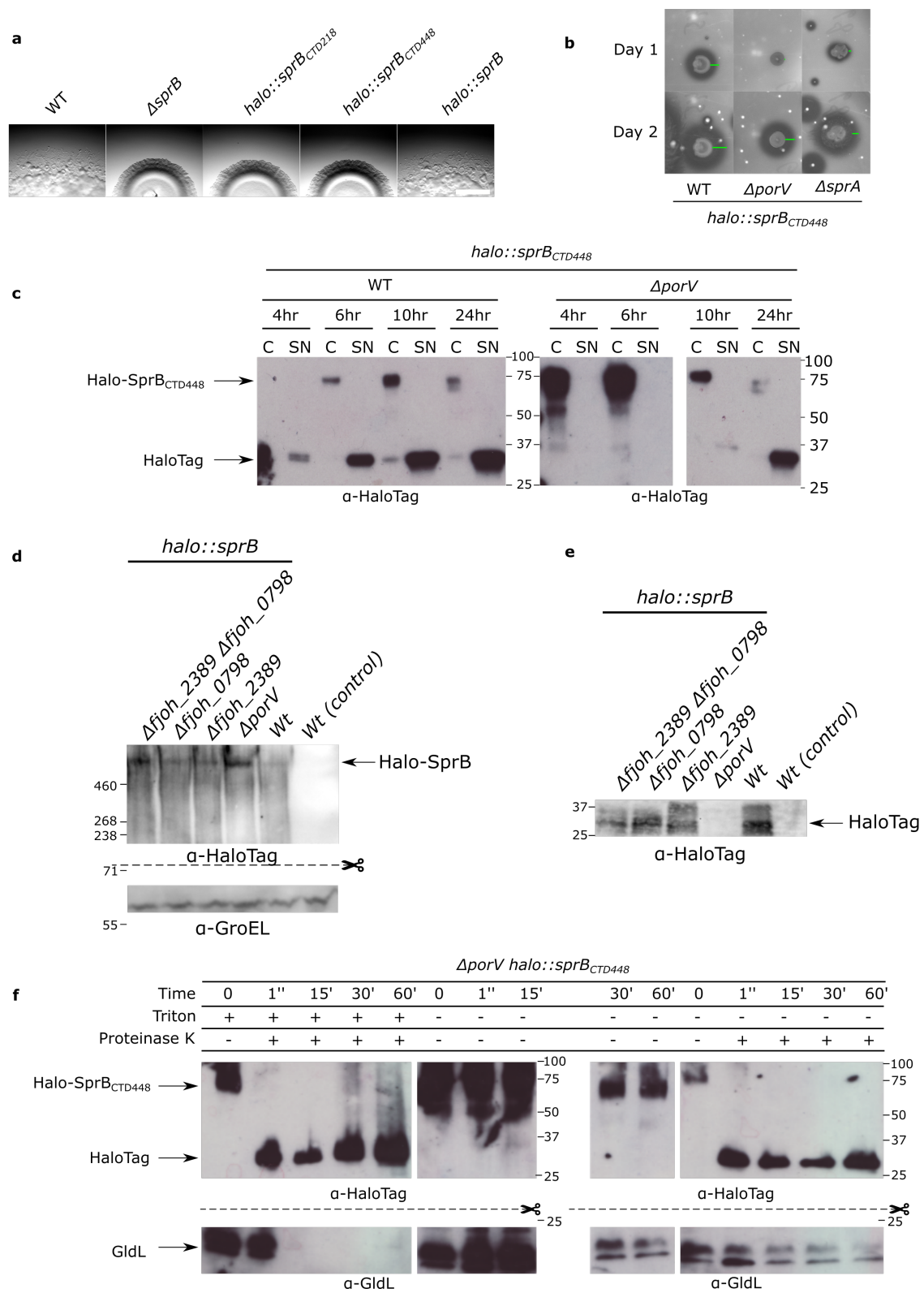


Figure 3.1: Characterisation of *halo-SprB* fusion strains. (a) Gliding motility was assessed by colony spreading on PY2 agar plates. The scale bar represents 3 mm. (b) Proteolytic activity was assessed by growth on MYA plates containing 30% skimmed milk. Colony clearing zones are indicated with green bars. The plates were imaged after 1 and 2 days. The scale bar represents 3 mm. The experiment was repeated two times. (c) Effect of *porV* deletion on the stability of Halo-SprB_{CTD448}. Samples were taken at the indicated time points during the growth of the cells in MM, separated into a cell (C) and supernatant (SN) fractions, and subjected to immunoblotting using HaloTag antibodies. The experiment was repeated 3 times. (d-e) Stability of Halo-SprB in different genetic backgrounds. Cells (d) and culture supernatants (e) were assessed by immunoblotting with HaloTag antibodies following growth in MM. GroEL is a cytoplasmic protein used as a loading control. Both blots were repeated 3 times. (f) Accessibility of Halo-SprB_{CTD448} to externally added Proteinase K. Cells were treated with Proteinase K or the detergent Triton X-100 as indicated. Samples for analysis were collected at the indicated times after reagent addition, Proteinase K was inactivated by addition of PMSF and boiling in SDS buffer, and analysed by immunoblotting. The cytoplasmic membrane protein GldL acts as a control for the impermeability of the outer membrane to proteinase K. The experiment was repeated 3 times.

3.2.2 2-dimensional single-particle tracking of Halo-SprB in immobilised cells as a tool for studying gliding motility

Using the $\Delta porV$ genetic background I was able to achieve a high labelling density of helically moving SprB adhesins. Since SPT is difficult when cells contain multiple fluorescent molecules I changed the labelling dye to photoactivatable JaneliaFluor646 with a HaloTag ligand (PAJF646-Halo). This enabled me to use photoactivation to turn on the fluorescence of 1-2 molecules at a time in each cell. This approach allowed me to follow multiple successive single-molecule trajectories in each cell with each track lasting several s. I was able to extend the time a molecule can be observed to >1 min by strobing the

laser and interpolating between images, taking advantage of the fact that SprB moves in a defined path.

By imaging the cells on agar pads as described in 2.4.1 it was possible to trap the cell body between the glass cover slide and the agar pad with enough force to prevent the cells from gliding. Importantly, these conditions do not prevent SprB movement. Thus this cell mounting method makes it possible to monitor the movement of SprB adhesins independent of cell body motion.

Although Halo-SprB molecules could be clearly seen by the eye to be moving in helical trajectories, multiple immobile foci were also observed with the relative proportions of the two types of behaviour subject to day-to-day variation. Some of the foci ($\sim 1/3$) were immobile and a few (~ 1 in 20) moved fast with apparent Brownian motion, indicative of a free HaloTag. To only analyse the helical tracks, I manually sorted the tracking data by inspecting a plot of the X-Y coordinates, speed, and position along the long axis of the cell. An example trajectory of Halo-SprB moving helically, representing the type of data extracted from the tracking is shown in Figure 3.2.

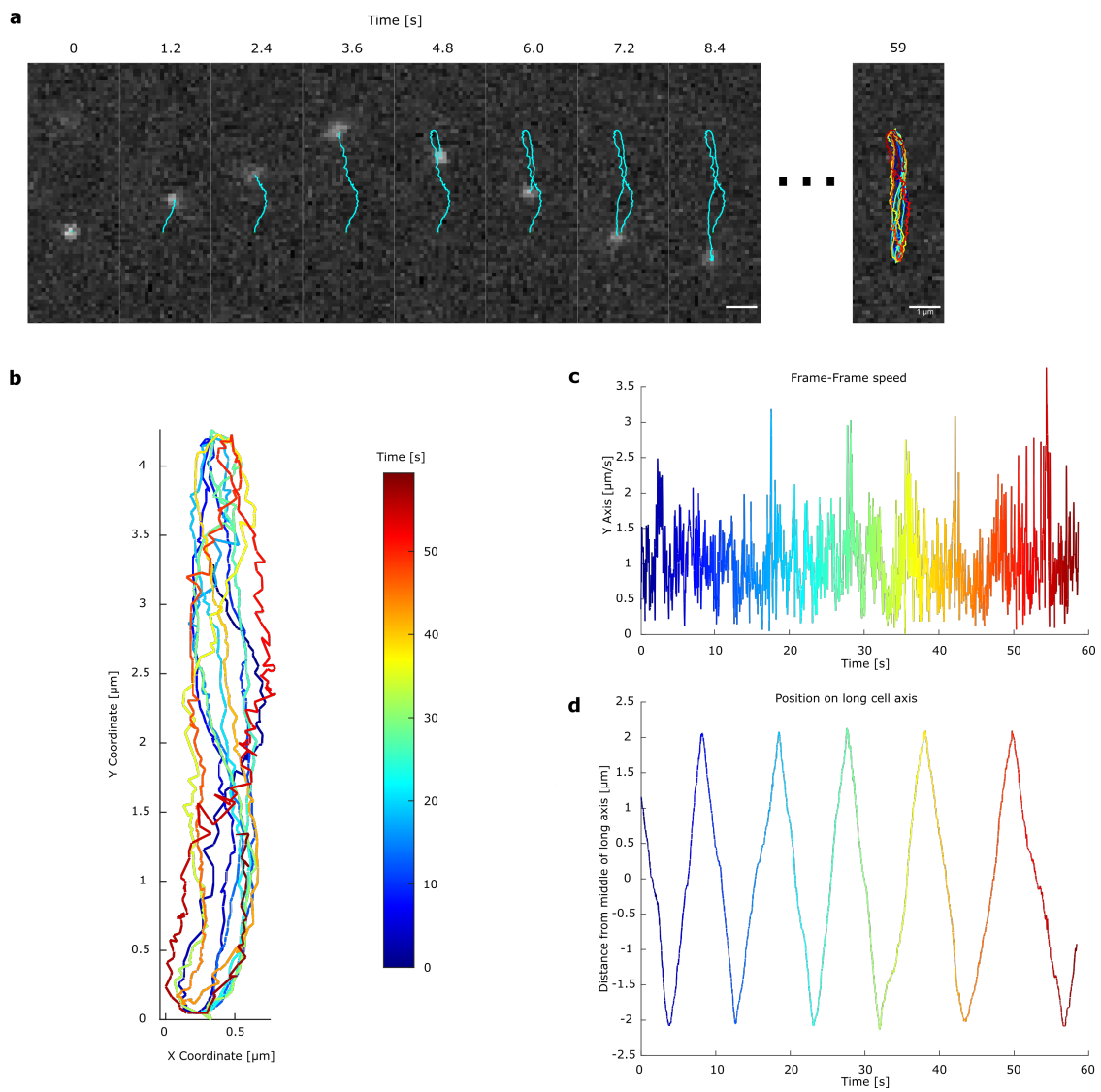


Figure 3.2: Halo-SprB single-particle tracking provides long tracks with high localisation precision. *halo::SprB ΔporV* cells were grown as described in 2.4.1 and labelled with 10 nM PAJF646-Halo. The cells were spotted on PY2+1% agarose pads and imaged as described in 2.4.2 using a 1 on 2 off strobing frequency. **(a)** A montage showing every 20th frame from the resulting video. Fluorescent Halo-SprB is seen as a white spot with tracking overlaid in cyan. The right-most frame shows the last frame of the movie with temporal colour-coding from blue to red. The scale bar indicates 1 μm . **(b)** The helical track from **(a)**. The colour bar gives the temporal coding for all panels in the figure except the cyan overlays in **a**. **(c-d)** Data obtained from the track shown in **(a-b)**. **(c)** Frame-by-frame speed was calculated using the euclidean distance between sequential (x,y) coordinates. **(d)** The position along the long axis of the cell was calculated by mapping all coordinates onto a line drawn from the two extreme points at each end of the track.

The methodology described above revealed several new findings regarding the track topology and dynamics. First, the SprB molecules do not move on a single closed loop as suggested in current models (Shrivastava, Berg, 2020) but instead trace a different path each time they traverse the length of the cell (Figure 3.2 **b**). By looking for deviations from the triangular wave that characterises the change in position along the long axis of the cell (Figure 3.2 **d**) it is apparent that the movements of the adhesin molecules sometimes reverse mid cell or the adhesin moves in small loops. An example of an adhesin moving in loops can be seen in Figure 3.3 which shows a circular loop occurring at both cell poles. The presence of reversals and local loops reveals a more complex movement pattern of adhesin movement, and thus of the organisation of the underlying tracks than currently appreciated. Occasionally, cells with multiple molecules labelled at the same time had one molecule immobile and one that moved. In these cases, there was no rolling or other movements of the immobile molecule, suggesting that the cells were still in the agar.

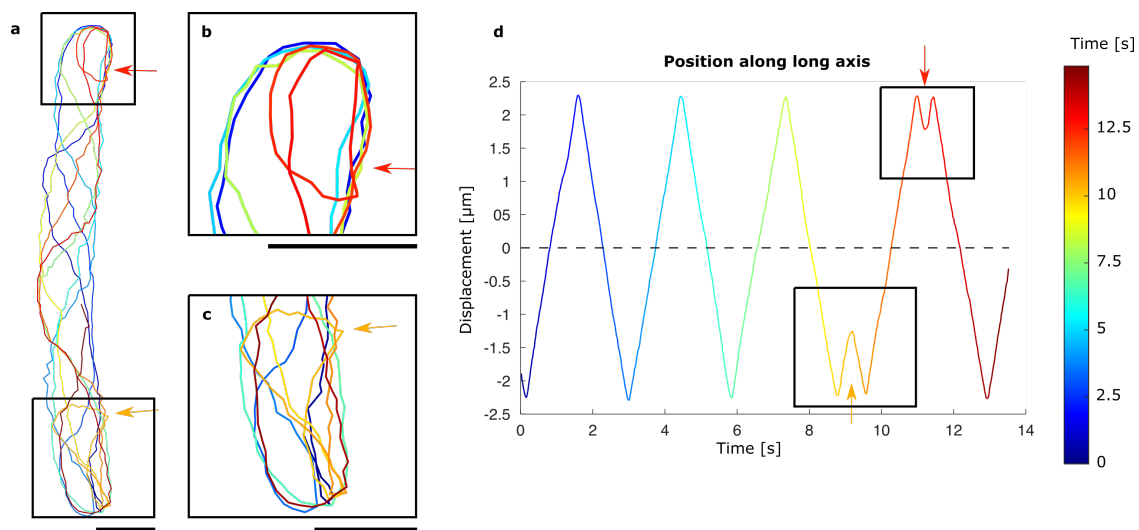


Figure 3.3: Adhesins can move in small loops. *halo::SprB* Δ *porV* cells were grown as described in 2.4.1, labelled with PAJF646-Halo and imaged as described in 2.4.2. (a) An example trajectory of Halo-SprB showing small track loops (b-c) near both poles of the cell with temporal colour-coding from blue to red. The scale bar in a-c indicates 500 nm. (b) Zoom of one of the pole loops. (c) Zoom of the other pole loop. (d) The position along the long axis of the cell in (a) calculated by mapping all coordinates onto a line drawn from the two extreme points at each end of the track. Deviations from the canonical helical track can be identified as deformities in the regular sawtooth shape of the plot. Loops shown in b-c are indicated by a black box. Arrows indicate when the adhesin is running along the small track loops.

In addition to topological information, SPT also revealed the dynamics of movement. Changes in the speed of moving adhesins were frequently observed with both increases and decreases in speed seen. The speed of a single molecule could vary from immobile to moving at $\sim 2 \frac{\mu\text{m}}{\text{s}}$. Figure 3.4 shows an example of a Halo-SprB molecule slowing down while continuing to move in a helical trajectory. Because the track was hypothesised to comprise a single connected track, I was surprised to find that each Halo-SprB molecule was essentially independent with respect to the speed of movement. When observing cells

with more than 1 fluorescent adhesin it frequently happened that the SprB molecules were able to move at different speeds within a single cell as shown in the example in Figure 3.4 **c-d**.

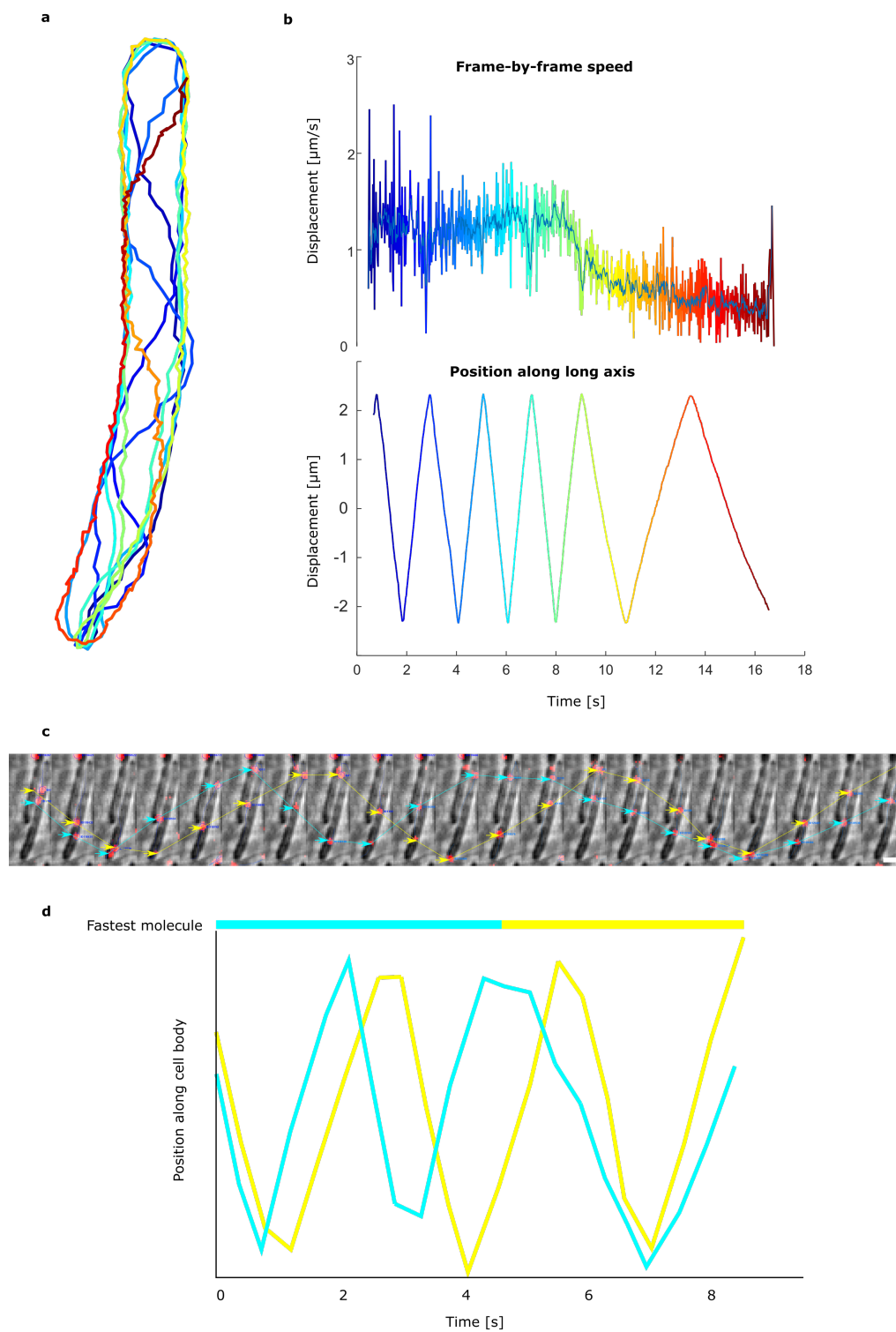


Figure 3.4: The speed of adhesin movement varies independently of other adhesin molecules in the cell. **(a)** A trajectory of Halo-SprB slowing down over time. Cells producing Halo-SprB were grown as described in 2.4.1 and imaged on agar pads as described in 2.4.2 with 1 on 3 off strobing. **(b)** Frame-by-frame displacement and position along the long axis of the cell for the adhesin in **(a)**. The adhesin slows down while keeping a helical trajectory. **(c)** Analysis of two fluorescent Halo-SprB molecules moving in the same cell. Cells of a similar genetic background were grown and imaged as described for **a**. Panels show every fifth frame from a 100-frame sequence. The tracks of the two molecules are shown in cyan and yellow. The scale bar represents 1 μm . **(d)** Graph of the position of the two molecules from **c** over time, with a bar on top indicating which molecule is moving faster.

3.2.3 Application of 2-dimensional single-particle tracking of Halo-SprB to assess the proton motive force harnessing efficacy of the GldLM complex

The cytoplasmic membrane GldLM proteins are responsible for harnessing the PMF to power both gliding motility and Type 9 protein transport. The structure of the GldLM complex was solved by Hennell James et al. (2021). Based on this structure, several polar residues were identified within the transmembrane part of the complex that might be involved in converting proton flow to mechanical motions. The importance of these residues to the GldLM function was tested by point substitutions (Hennell James et al., 2021). Some of these substitutions partially affected colony spreading on PY2 agar plates. A reduction in colony spreading on PY2 agar plates can arise not only from defects in gliding motility but also from deficiencies in Type 9 export of the adhesins required for gliding. Since both these processes require the GldLM complex untangling the functional effects

of the point substitutions is not straightforward. However, I was able to directly assess the effects of the substitutions on gliding by taking advantage of the SPT methodology described above to measure the speed of adhesin movement (Figure 3.5). The poorly-spreading variants that were analysed were GldL Y13F, GldL H30A, GldL K27A, GldM R9K, and GldM Y17F. From manual inspection of the resulting videos, it was clear that different substitutions had a different effect on the speed at which the adhesin travelled. The speed of each measured track was averaged and then pooled to provide a single mean speed value for each biological replicate. Both with and without pooling of the data, the resulting boxplots revealed heteroskedasticity of the data. For this reason, I employed a generalised least squares (GLS) model that allows different variances for each strain. Testing the model with *a priori* defined contrasts, and adjusting the obtained *p*-value using the Shaffer method (Shaffer, 1995), all pairwise comparisons of mutants with the parental strain displayed significant ($p < 0.001$) difference in their means. Thus each substitution had an effect on the speed of SprB protein movement, suggesting that the substitutions are important for the efficient harnessing of protons.

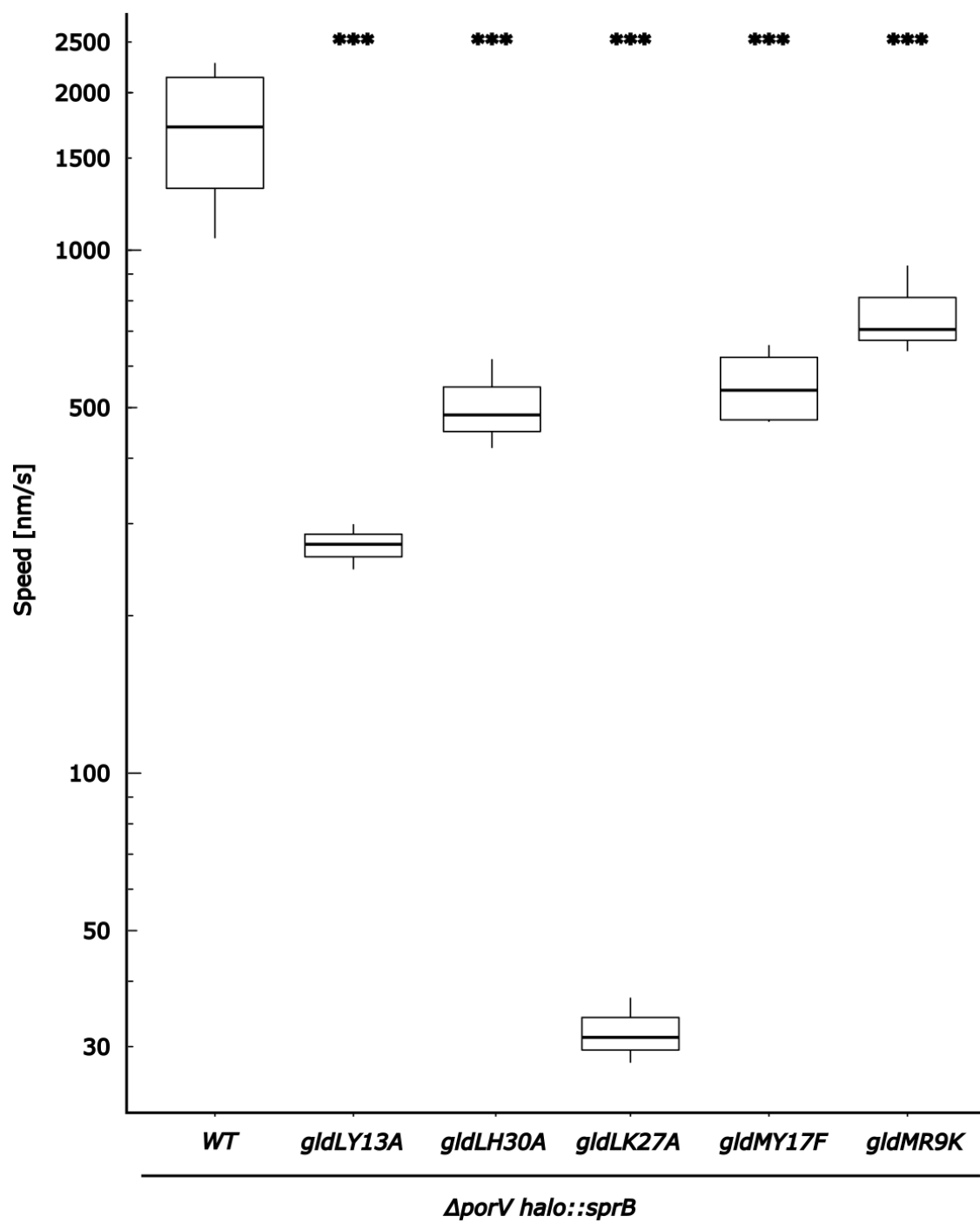


Figure 3.5: single-particle tracking of Halo-SprB in GldLM variants measures the efficiency of proton motive force harnessing. Speed of Halo-SprB movement was assessed for strains expressing Halo-SprB in different genetic backgrounds carrying a point mutation in either *gldL* or *gldM*. The strains were grown as described in 2.4.1, labelled with 10 nM PAJF646-Halo, deposited on agar pads, and imaged as described in 2.4.2 using a 1 to 25 light:dark cycle for the *gldLK27A* mutant and a 1 to 4 light:dark cycle for the remaining strains. The tracking data were sorted manually to isolate helical tracks and the average speed was calculated for each track and then averaged for each biological repeat. The total number of tracks for each strain in the boxplot listed from left (WT) to right (*gldMR9K*) were: 321, 499, 3549, 372, 1475, 429. Each strain was compared to the parental strain, *halo::SprB* Δ *porV*. The data were modelled with a generalised least squares model, allowing a different variance for each strain, and contrasts for pairwise comparisons against the parental strain were defined *a priori*. The obtained *p*-values were corrected with Shaffer’s method (Shaffer, 1995). ***= $p < 0.001$.

Manual inspection of the tracking data for the *gldLM* point mutants suggested that the circle-loop behaviour seen with the parental strain (discussed above) was more common in the mutant strains. These circles had a diameter of ~ 300 nm (Figure 3.6, **a-b**) and the particles moved with constant speed (Figure 3.6, **c**). I counted and calculated the frequency of the occurrence of loops relative to helical tracks (Figure 3.6 **d**). By employing a general linear model that corrects for the number of tracks in each biological replicate, I found that the frequency of loops was significantly higher in the *gldLM* point mutants than the parental strain, but was not correlated with the observed speeds of adhesin movement (Figure 3.6 **d**). Shrivastava et al. (2015) demonstrated an adhesin shearing technique that generated cells that were stuck to a glass slide via SprB antibodies and spinning with a frequency of about 1 Hz. The authors also showed that adding SprB antibody-coated $0.5 \mu\text{m}$ latex beads to sheered cells resulted in the beads spinning at 1 Hz around a ~ 300 nm diameter circle. The diameter of the spinning bead is similar to the size of the circle-loops

I observe. To obtain the angular frequency of the circle-loops, I first smoothed the data with a 5-frame window before converting the positional coordinates into angles relative to the centre of the loop and then calculated the corresponding Fourier spectrum. A clear singular peak was seen in the magnitude of the Fourier transform spectrum proving that the angular frequency was relatively constant (Figure 3.6 f). The major wave component from the Fourier transform was calculated (Amplitude: 41 °, Frequency: 1 Hz, and Phase: 1.81) and used to plot a cosine wave on top of the angular position (Figure 3.6 f). A comparison of the two waves suggests the estimated frequency is close to that of the observed. Thus, the attenuated harnessing of protons seems to affect the topology of SprB trajectories. It is not known why this occurs, but given that cells exposed to viscous shear also display similar behaviour, it suggests that the behaviour is indicative of a general malfunction of the gliding machine.

Figure 3.6: Small loops are found more often in GldLM variants. **a-b** An example of a small circular loop viewed in 2D (**a**) and in 3D with time as the 3rd dimension (**b**). (**c**). Frame-by-frame displacements were plotted for the trajectory shown in (**a-b**). The colour bar to the right indicates the time in **a-c**. (**d**) The frequency of loop occurrences was plotted for the WT and GldLM PM strains. The data were modelled with a generalised linear model using a binomial distribution and accounting for the number of tracks in each dataset. Comparisons were made using *a priori* defined pairwise contrasts and the obtained *p*-values were corrected according to Shaffer's method (Shaffer, 1995). *** = $p < 0.001$. (**e**) The angular position of the moving particle relative to the centre of the loop was calculated, Fourier transformed and the magnitude spectrum plotted. The magnitude spectrum shows the decomposition of the sinusoidal angular position, with the component waves amplitude shown on the Y-axis and the frequency on the X-axis. (**f**) The peak from the Fourier spectrum shown in (**e**) was used to estimate the most prominent wave of the angular position data. This major wave component was then plotted (red) on top of the original angular position data (blue).

3.2.4 3D single-particle tracking of SprB reveals insights into the track topology

2D SPT of SprB only provides a projection map of the true 3D track network. To obtain a fully defined picture of the tracks, I implemented 3D SPT. This was achieved by employing a cylindrical lens that distorts the PSF as a function of the position in Z relative to the focus plane. The Z-position was then interpreted by comparing the distortion to a pre-calculated reference that was created by collecting Z-stacks of reference beads. The quality of the 3D SPT of Halo-SprB was further improved by applying a moving average smoothing of the coordinates in all 3 dimensions, using a 5-frame window. This window-size result in a slight reduction of the length of the track, but otherwise seems to conserve most of the information of the track topology while greatly reducing the artefacts of

localisation noise (Figure 3.7). A dataset of 179 tracks was collected with the *halo::SprB* $\Delta porV$ strain and used for analysis and method development as described below.

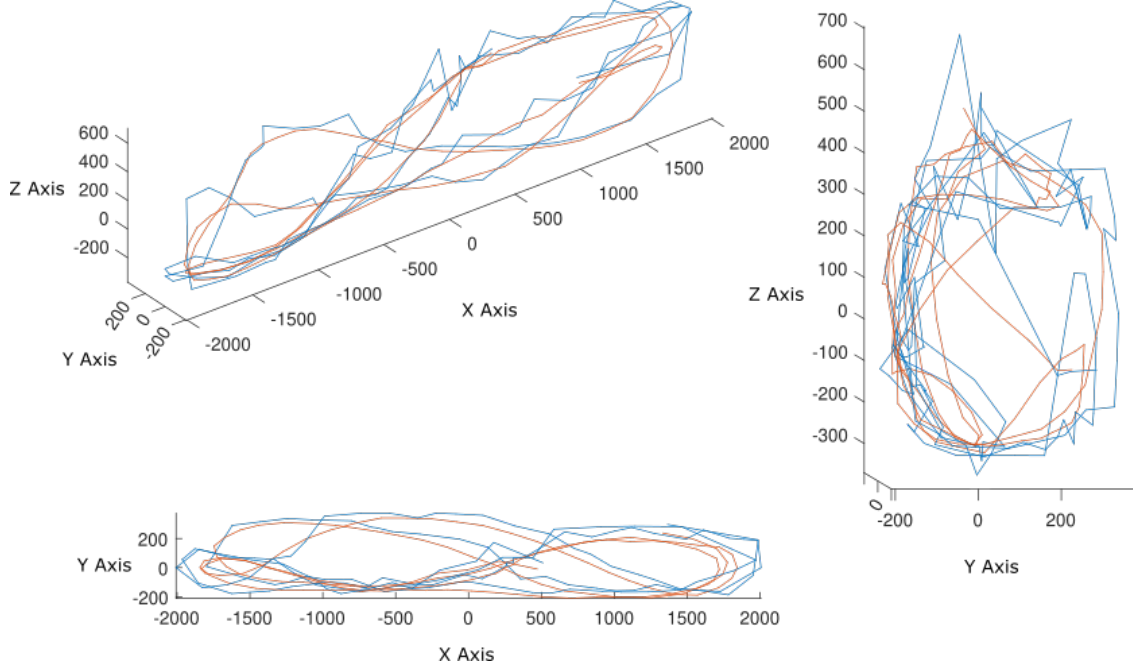


Figure 3.7: A representative 3-dimensional single-particle tracking trajectory of Halo-SprB. *halo::sprB* $\Delta porV$ mutant cells were grown as described in 2.4.1, labelled with 0.01 nM JF646-HaloTag and imaged as described in 2.4.2 and strobed with a 1 on 4 off frequency. Molecules were identified and tracked as described in 2.2. The blue line represents raw data and the orange is the smoothed version of the blue, using a window of 5 frames. The duration of the track is 141 frames, equivalent to 11.28 s. All axis units are in nm.

Taking the transformed and smoothed 3D data that has the long axis of the cell aligned with the X axis and all dimensions centred in 0, I calculated the pitch angle in 3D using equation with a window of 5 frames 3.1.

$$\cos\theta = \frac{\vec{dr1} \cdot \vec{dr2}}{\|\vec{dr1}\| \|\vec{dr2}\|} \quad (3.1)$$

In this equation $d\vec{r}1$ refers to the point-to-point vector of the data (Equation 3.2) and $d\vec{r}2$ is the point-to-point change in the x-axis coordinate with Y and Z set to zero (Equation 3.3).

$$d\vec{r}1 = \begin{bmatrix} x_5 - x_0 & y_5 - y_0 & z_5 - z_0 \\ x_6 - x_1 & y_6 - y_1 & z_6 - z_1 \\ \vdots & \vdots & \vdots \\ x_{i-1+5} - x_i & y_{i-1} - y_i & z_{i-1} - z_i \\ x_{i+5} - x_i & y_{i+5} - y_i & z_{i+5} - z_i \end{bmatrix} \quad (3.2)$$

$$d\vec{r}2 = \begin{bmatrix} x_5 - x_0 & 0 & 0 \\ x_6 - x_1 & 0 & 0 \\ \vdots & \vdots & \vdots \\ x_{i-1+5} - x_i & 0 & 0 \\ x_{i+5} - x_i & 0 & 0 \end{bmatrix} \quad (3.3)$$

the angle between $d\vec{r}1$ and $d\vec{r}2$ should in this case be the pitch angle, θ , relative to the long (x) axis of the cell.

The histogram of frame-to-frame angles, pooled across the 179 tracks, was fitted with a single Gaussian (equation 3.4) to estimate the mean and standard deviation of the peak. This had an adjusted R-square value of 0.9662 (Figure 3.1, red line). From the fitted Gaussian, the angle was estimated to be $17.62^\circ \pm 2.73^\circ$ (SD), which is not far from the $19.0 \pm 11.5^\circ$ reported by Nakane et al. (2013).

$$f(x) = Ae^{\left(\frac{(x-\mu)^2}{2\sigma^2}\right)} \quad (3.4)$$

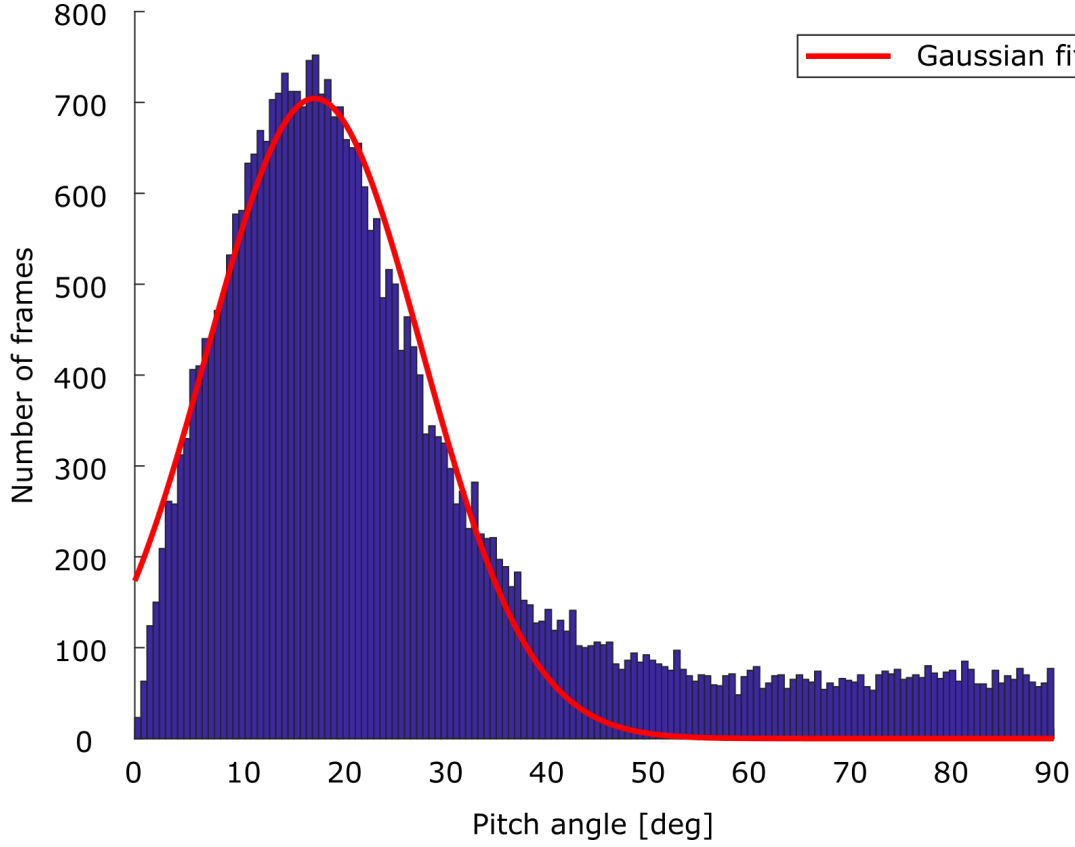


Figure 3.8: Histogram of frame-to-frame helix pitch angle from a data set of 179 individual 3D tracks of moving adhesins. 3D SPT data from 3 biological repeats using the *halo::sprBΔporV* strain were pooled and analysed to find the pitch angle relative to the long axis of the cell. The red line indicates the fitted Gaussian equation to the data, which has an adjusted R-square value of 0.9662.

Using the same 3D data set, I measured the frame-by-frame 3D displacement using equa-

tion 3.5.

$$3D \text{ Displacement} = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2} = \sqrt{\Delta x^2 + \Delta y^2 + \Delta z^2} \quad (3.5)$$

One major and two minor peaks were observed. The minor peaks are probably the result of particles that are either moving helically for a part of the duration and then stop or that are wrongfully assigned to another Brownian moving molecule for a part of their trajectory. A single Gaussian fit was made using equation 3.4 (adjusted R-squared: 0.9662). Although the fit was slightly confounded by the two minor peaks, visual inspection suggests the mean is not far from the true mean of the major peak. From this Gaussian fit, the speed of adhesin movement is estimated to be $1.74 \frac{\mu m}{s} \pm 0.89 \frac{\mu m}{s}$ (SD).

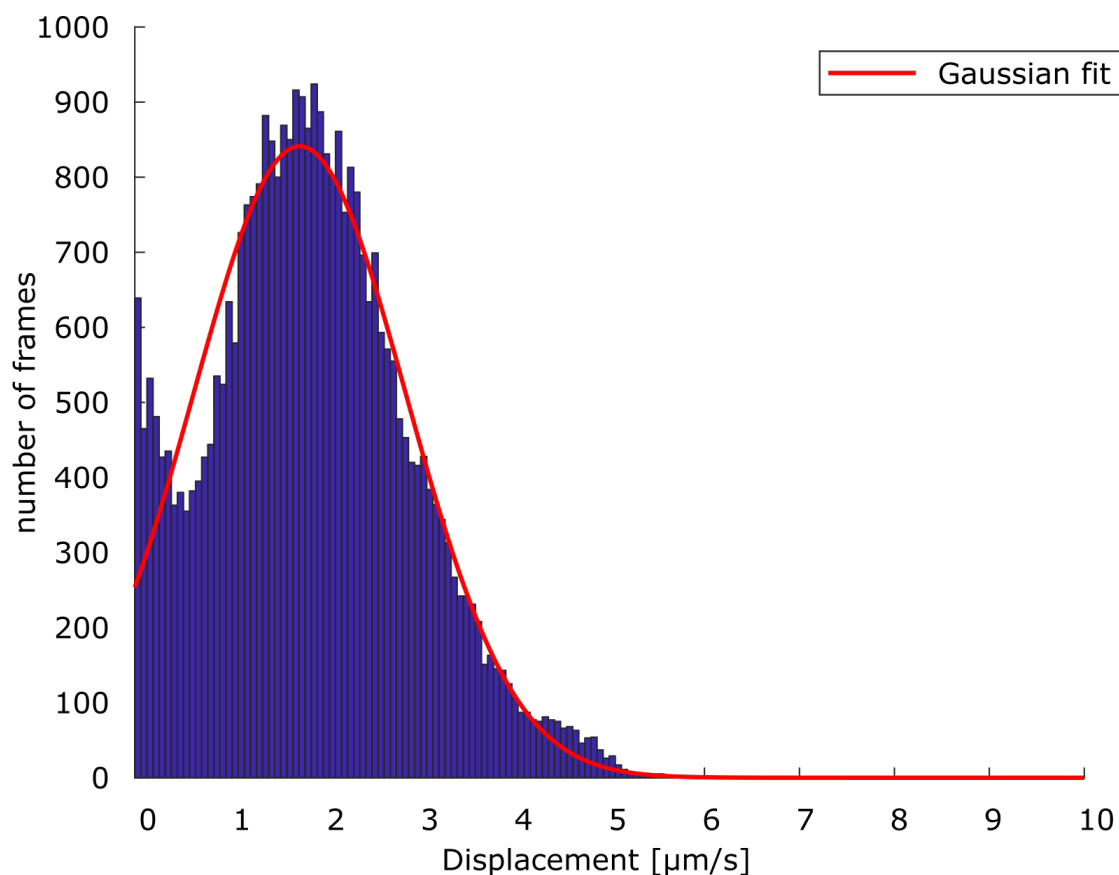


Figure 3.9: Histogram of frame-to-frame 3D displacement from a data set of 179 individual tracks, collected from 3 single-particle tracking imaging experiments of the *halo::sprB* $\Delta porV$ strain. The red line represents a fitted Gaussian (adjusted R-square value: 0.9662).

3.2.5 Automatic classification of single-particle tracking data to quantify the proportion of tracks moving in a helix

Fluorescently labelled Halo-SprB molecules were variously observed to display Brownian, immobile or directed (helical) motion. To quantify the proportion of molecules moving

helically, I trained a random forest machine learning (ML) algorithm to discern between these types of movement automatically.

Before sorting the tracks automatically, however, I had to clean the SPT data from contaminants caused by spurious localisations from fluorescent 'dirt' in the agar pads. To minimise this background I had to restrict the analysis to localisations that are contained within cells. Therefore, I used a fluorescent membrane dye (SynaptoGreen) to obtain data on the cell outlines. These outlines were then used to subset the localisations of a given field of view to only those that occurred within a cell boundary (Figure 3.10 **a**). This efficiently removed all dirt from areas outside cells and provided tracks from within the cell bodies that were either moving helically or not (Figure 3.10 **b**).

Helical tracks are characterised by their speed, periodicity, direction, and overall topology. Therefore, I designed the ML algorithm to distinguish the tracks based on these types of information. The speed was represented by the mean squared displacement (MSD). The MSD was calculated for each track by splitting the track in a $30(n)$ -frame window starting from each frame index, j (Equation 3.6). The distance of a starting point (Equation 3.7 $\vec{A}$) to all points from x_j to x_{j+n} (Equation 3.7 $\vec{B}$) were calculated (Equation 3.8 $\vec{D}$) and differentiated (Equation 3.8 $\vec{D}'$) to obtain the MSD at all lag-times and the slope of that line, respectively. The average slope was used as the MSD for each j and stored in a new vector (Equation 3.9 MSD).

$$n = 30, j = 1, 2 \dots \#frames \text{ in track} - n \quad (3.6)$$

$$\vec{A} = n \text{ elements} \left\{ \begin{bmatrix} x_j \\ x_j \\ \vdots \\ x_j \end{bmatrix} \right\}, \vec{B} = \begin{bmatrix} x_j \\ x_{j+1} \\ \vdots \\ x_{j+n} \end{bmatrix} \quad (3.7)$$

$$\vec{D} = \sqrt{A^2 - B^2}, \vec{D}' = \begin{bmatrix} D_2 - D_1 \\ D_3 - D_2 \\ \vdots \\ D_n - D_{n-1} \end{bmatrix} \quad (3.8)$$

$$MSD(j) = \frac{1}{n} \sum_{i=1}^n D'_i = \frac{D'_1 + D'_2 + \cdots + D'_n}{n} \quad (3.9)$$

The MSD calculation was extended to encompass d dimensions by modifying $\vec{A}$, $\vec{B}$, and $\vec{D}$ (Equation 3.10 and 3.11).

$$d = 3, \vec{A} = n \text{ elements} \left\{ \begin{bmatrix} x_j & y_j & z_j \\ x_j & y_j & z_j \\ \vdots & \vdots & \vdots \\ x_j & y_j & z_j \end{bmatrix} \right\}, \vec{B} = \begin{bmatrix} x_j & y_j & z_j \\ x_{j+1} & y_{j+1} & z_{j+1} \\ \vdots & \vdots & \vdots \\ x_{j+n} & y_{j+n} & z_{j+n} \end{bmatrix} \quad (3.10)$$

$$\vec{D} = \sqrt{\sum_{i=1}^d A_{*,i}^{\vec{}}^2 - B_{*,i}^{\vec{}}^2} = \sqrt{A_{*,1}^{\vec{}}^2 - B_{*,1}^{\vec{}}^2 + A_{*,2}^{\vec{}}^2 - B_{*,2}^{\vec{}}^2 + A_{*,3}^{\vec{}}^2 - B_{*,3}^{\vec{}}^2} \quad (3.11)$$

To incorporate the information on the direction of the moving molecule, I calculated the pitch angle according to Equation 3.1.

Because both the coordinate position and pitch angle had a characteristic periodicity, I calculated the 3 major frequency components (frequency, amplitude, and phase) of each time series (Smoothed X, Y, and Z coordinates and pitch angle) with discrete Fourier transform using the fast Fourier transform Matlab function, *fft*.

The ML program requires a table of features that characterises a track, wherein each column is a feature and each row is a track. Therefore, the characteristic time-series features (MSD and pitch angle) had to be decomposed into single value entities. To accomplish this I calculated the mean, variance, minimum, and maximum values and used those to represent the time series. Because I selected the 3 largest wave components from the Fourier transform, I could use those values without modification.

The dimensions and shape of the track also provide information useful for the classification of helical tracks. Immobile molecules will have very small bounding boxes and tracks that move in confined areas of the cell will be less likely to adopt an elliptical shape. Therefore, I calculated the bounding box as well as an elliptical fit to the data. While a bounding box is more robust, an elliptical fit more accurately represents the desired shape and tolerates a few outlier points better. The bounding box was simply defined as a box encompassing the minimum to maximum coordinate values in each dimension. The ellipse (Equation 3.12) was fitted using a least-square estimation method without weights (Gal, 2003).

$$Ellipse = ax^2 + bxy + cy^2 + dx + ey + f = 0 \quad (3.12)$$

Again, single value decomposition was necessary to obtain features that could be used with the ML algorithm. The fitted ellipse was represented by the estimated parameters as well as the residual sum of squares from the distance between the fitted ellipse and the data points (Ma, 2010). Furthermore, the minimum, maximum, mean, and variance of the distance between the fitted ellipse and the data were calculated and included as features in the ML program.

To train the ML algorithm, I manually classified tracks as helical or not and used the dataset (5392 tracks) for the training of the model. The manual classification was done by inspecting a multi-figure image of each track that showed the speed, position along the long axis of the cell, and smoothed and unsmoothed track. These features made it easy to discern between a helical and non-helical track. After training, the performance of the ML algorithm was evaluated using a confusion matrix. A confusion matrix indicates the correct and incorrect classifications done by the ML algorithm, giving a measure of how successful it was in reproducing the manual classification. The confusion matrix can be seen in Figure 3.10. A majority of tracks were correctly classified as non-helical, whereas only 86.6 % of the helical tracks were classified as helical by the ML algorithm. This lower success of helical classification is likely because tracks with very slow-moving molecules were difficult to classify as either helical or not. Indeed, the most common error seems to be wrongly classifying a helical track as non-helical (13.4%) whereas wrongly classifying a non-helical track as helical rarely occurred (0.7%). This suggests that the ML algorithm is useful in quantifying large numbers of molecules as helical or not, whereas, in more qualitative assays, manual inspection post-sorting would be required for precise results.

Following the classification of the data, around 55% of adhesins were reported to move in helical tracks. This value is higher than the reported $\sim 35\%$ of labels that moved, observed by Kulkarni et al. (2019). Significant day-to-day variation was observed, however, so it is hard to say if this difference is significant or not.

In summary, 3D SPT provides high-quality data on the helical movement of SprB. Using this data with the trained random forest ML algorithm, fast classification of thousands of tracks as either helical or not can be achieved.

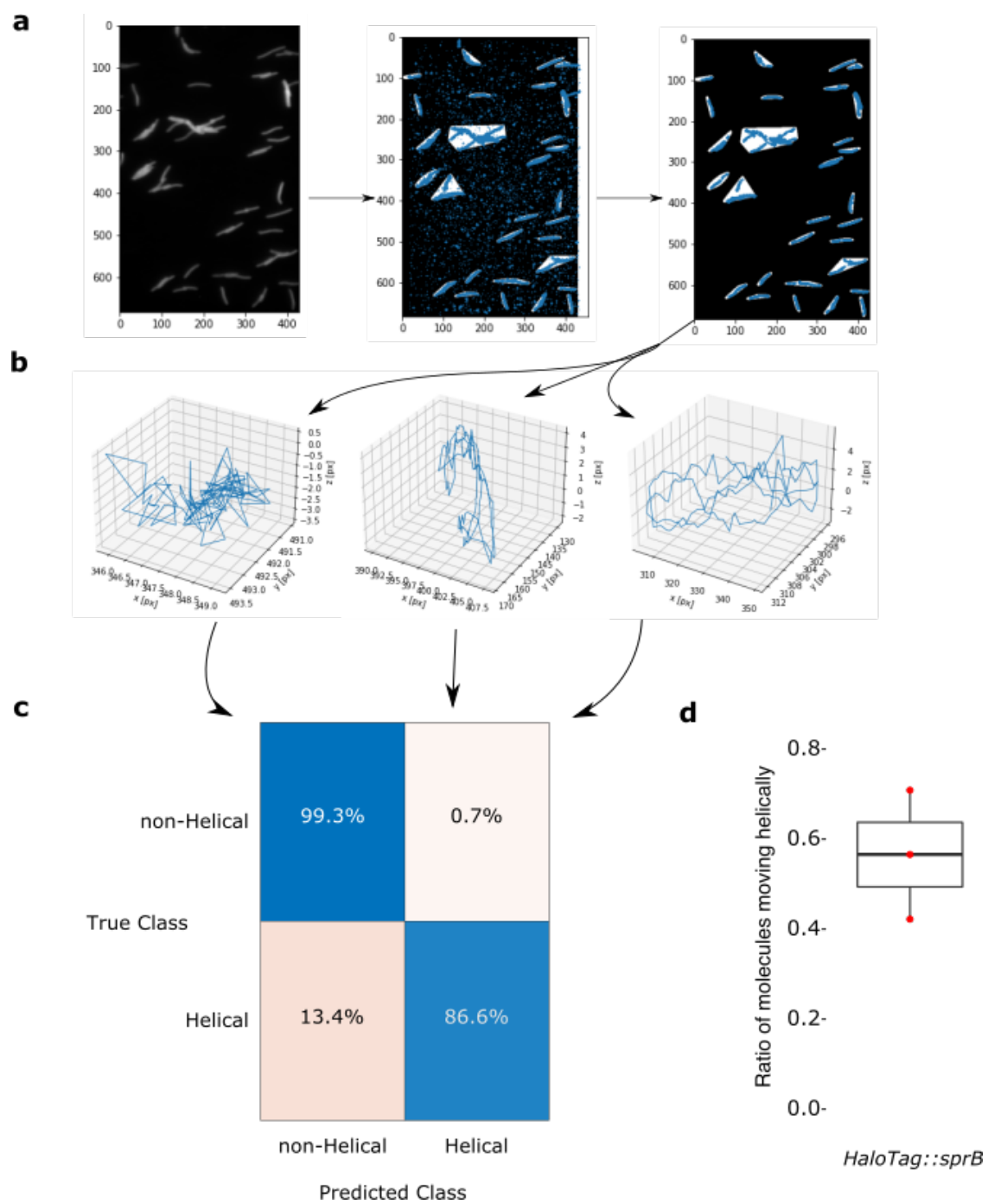


Figure 3.10: Automated classification of helical tracks. *halo::sprB* Δ *porV* cells were grown as described in 2.4.1, labelled with 0.01 nM JF646-Halo dye and imaged as described in 2.4.2 using 1 on 4 off strobing. **(a)** Cell segmentation using the membrane dye data was used to filter the localisations (blue). Scale bar on the edge of frames are measured in pixels. **(b)** The tracks were sorted using a supervised random forest machine learning algorithm, implemented in the Matlab programming language. **(d)** The ratios of helical tracks were plotted as a boxplot with each datapoint representing a biological replicate. The pixel size in **a** and **b** is $116.9 \frac{nm}{pixel}$

3.3 Discussion

SprB is the major adhesin involved in *F. johnsoniae* gliding motility. Previous studies have followed the movement of SprB on the cell surface with SprB antibody-coated beads Nakane et al. (2013) or SprB antibodies labelled with either fluorophores (Kulkarni et al., 2019) or gold beads (Shrivastava et al., 2016). In this chapter, I presented a new imaging approach that allows SprB movements to be studied in greater detail. By fusing the self-labelling enzyme HaloTag to SprB I was able to label the adhesin with bright and photostable dyes. Both full length and truncated Halo-SprB fusions were found to suffer significant proteolysis in the WT genetic background. However, deletion of *porV* reduced the proteolytic capability of *F. johnsoniae* and also resulted in significantly reduced cleavage of the fusion proteins (Figure 3.1). PorV is a major shuttle protein required for the T9SS-secretion of all Type A CTD containing proteins. I did not identify which particular proteases were responsible for the degradation of SprB but showed that this did not involve the proteases Fjoh_2389 or Fjoh_0798 alone or in combination.

Previous attempts at analysing the movements of SprB have been hampered by an inability

to separate the movement of the cell body from that of the protein. By using a simple agar pad setup that traps the cells between the glass slide and agar pad and prevents the cell body from moving, I was able to study SprB movement without this complication. By using the far-red dye JF646-Halo, or the photoactivatable version PAJF646-Halo, as a HaloTag label, I could capture trajectories of a single SprB molecule over >1000 frames. The length of time over which measurements could be carried out was further optimised by strobing the laser to reduce the photobleaching rate rather than continuously illuminating the sample. This technique allowed me to resolve very slow, as well as very fast, moving proteins by adjusting the strobing frequency accordingly. For example, tracks were resolved for very slow-moving adhesins in the *gldLK27A* gliding motor mutant which moved with a mean speed of $\sim 30\text{nm/s}$. This is more than 50 times slower than the movement of the adhesin in the parental strain. Nevertheless, the adhesin still moved in a helical track suggesting that the power output of the motor complex does not have a strong effect on the track topology.

Although most molecules moved helically in the *gldLM* mutants, I did observe up to $\sim 2\%$ of the helical tracks forming small loops. These loops were consistent in the diameter and frequency of the rotation and are likely the basis of the same observation reported by Shrivastava et al. (2015). The loops were also in the WT, although less frequently. I hypothesise that the loops are an inherent challenge that arises from the nature of the machine and that the cells have evolved to avoid such looping behaviour. If that is true, it would explain why mutations in the motor or viscous shearing would disrupt the machine and form such loops more frequently, as such alterations disrupt the optimised function.

Shrivastava et al. (2015) proposed that the cell rotation around a point of fixation, likely to be caused by the same loop presented in this thesis, is a result of SprB that gets stuck in a single rotary motor. Given that the loop is large enough to cover the circumference of the cell body (~ 300 nm) this does not seem feasible. The GldKN ring (50 nm $\varnothing$) and rotating GldM (20 nm $\varnothing$) are much smaller than the loop and so I argue that a more likely explanation could be that the track forms a closed loop, keeping the SprB locked in the same path. In support of this, regular helically moving molecules could occasionally do a single or double loop, often near cell poles without any alteration or disruption. If SprB would get stuck in the motor, it seems less likely that this would occur without any change in speed or continuity of movement. Also, the two polar loops shown in Figure 3.3 have slightly different sizes, supporting a track deformation model wherein the loop is not dependent on a fixed size structure such as the T9SS complex. Occasionally, looping molecules were observed to enter and/or exit loops that lasted for several rotations. Thus suggesting that such a fixed loop either exists for a variable duration of time before breaking up or gets repaired, or that a moving track is stuck following a groove or path along e.g. the peptidoglycan that keeps it from breaking the loop. Interestingly, other SprB molecules were also observed to move past a pole with a looping molecule, supporting a model wherein tracks can cross paths.

To extend the precision of Halo-SprB tracking I expanded the experimental setup and analysis to include 3D information. The increase in spatial information allowed a more detailed analysis of the movement pattern of the SprB adhesin. The observed pitch angle ($17.62^\circ \pm 2.73^\circ$ (SD)) agrees with a previously reported value derived from 2D data (19.0

$\pm 11.5^\circ$ Nakane et al. (2013)). The observed speed in 2D (Figure 3.5) and 3D ($1.74 \frac{\mu m}{s} \pm 0.89 \frac{\mu m}{s}$ (SD)) also correspond to previously observed value derived from 2D data ($2.1 \frac{\mu m}{s} \pm 1.0 \frac{\mu m}{s}$ (Nakane et al., 2013)). The speed corresponds to the observed speed that cells move ($\sim 2 \frac{\mu m}{s}$) when they are not trapped in agar pads, suggesting the agar pad does not significantly affect SprB movement. It is not obvious how this is possible since the cell body should be in contact with both the agar and glass and therefore have plenty of surface area where the Halo-SprB protein should adhere and not move. One possibility is that the protein machinery defining the track topology reorients itself to avoid areas of the cell surface that are under pressure. This seems unlikely given the spread of tracks over the cell body as e.g. seen in 2D in Figure 3.2 and 3D in Figure 3.7. The cell body may be trapped with sufficient force to stop the SprB proteins from moving it or maintaining attachment to the surroundings, or it could be that the cell has pressure or load-sensing response that regulates the attachment of SprB under these conditions. This suggestion is reasonable given that SprB proteins moving forward in a translocating cell do not stick to the substratum, and so it is likely that there exists an attachment-modulating functionality in the gliding machinery.

Current models of *F. johnsoniae* gliding has suggested that there is a single closed track on which all SprB proteins are simultaneously moving (Shrivastava, Berg, 2020)(Nakane et al., 2013). However, the SPT data presented in this chapter show that this is incorrect. First, the trajectory of single SprB molecules that moved over the cell body several times did not follow the same path with each repetition. Second, multiple SprB proteins were able to move at different and varying speeds in the same cell. These observations do not

completely reject the hypothesis of closed-looped tracks, however, it requires there to be several tracks that each has a defined speed and topology. Although possible, it does become less likely since a single molecule would have to be able to switch between these tracks constantly to agree with the observation that a single molecule follows multiple tracks. Cell body movement was also ruled out by observing immobile molecules during 3D imaging. These immobile molecules did not move even when mobile SprB molecules would move around in a helix in the same cell, suggesting that there is no obscuring cell movement. Instead, I suggest an alternative hypothesis where short dynamic tracks formed by tread-milling GldJ connect stationary T9SS "power stations", a theory that allows for multiple speeds of SprB within the same cell. In this model, inspired by tread-milling MreB, the treadmilling nature of GldJ follow the membrane curvature and/or helical grooves in the peptidoglycan to form a helical track. The T9SS OM lipoprotein, GldK, interfaces with GldJ and transfers the energy required for adhesin movement. Thus GldJ grows actively with the energy from GldLM through GldK, and the growing track treadmills at least as far as the following T9SS complex. With varying frequency from day to day, about ~ 30 % of SprB were immobile. If GldJ treadmills as suggested, these immobile proteins could be examples where a treadmilling track fails to connect with a motor unit. Alternatively, the connection between GldJ and SprB is lost, resulting in SprB being stuck in the OM, awaiting a connection to a track. The former hypothesis seems more plausible since SprB has been observed to slow down and even move extremely slowly, suggesting they are often connected to the track despite little-to-no movement. If there is a lack of motor assemblies resulting from e.g. viscous shear or mutations reducing

the number of available motor units, that would decrease the likelihood of a treadmilling track finding a route between two motor units. If this is the case, and GldJ treadmilling is guided by membrane curvature, it would explain the increased likelihood of finding loops near the pole. Polar loops would be explained as a closed ring powered by a single motor unit.

The treadmilling hypothesis only provides weak arguments for why SprB trajectories are able to cross each other. Sometimes, SprB moved past other pole-looping SprB where the model would expect a constant small track had formed. It is not known how tracks can cross yet and future structural work will be invaluable in answering these questions.

Chapter 4

Proteins from the *sprB* operon

regulate adhesion to direct gliding

motility in *Flavobacterium*

johnsoniae

4.1 Introduction

The Type 9 secretion system (T9SS) and the associated gliding motility apparatus are unique to bacteria of the *Bacteroidetes* phylum (McBride, 2019). Gliding motility involves a corkscrewing movement of the rod-shaped cells towards one of the cell poles. Gliding is mediated by adhesin proteins that move in helical paths lengthwise along the cell surface.

Nakane et al. (2013) hypothesised that the adhesins must adhere to the substratum only during protein movement from the leading pole to the lagging pole, and therefore must subsequently release the substratum while continuing their movement from the lagging pole back to the leading pole. In this way, the cell makes a net movement in the direction of the leading pole.

The adhesins are secreted by the T9SS which also provides the energy required for their movement (Sato et al., 2010). The T9SS is composed of several core proteins including a proton motive force (PMF)-harnessing motor complex composed of GldLM, the periplasmic proteins GldKN and SprE, and the outer membrane (OM) proteins SprAT. SprA is a large β -barrel protein that forms the OM pore of the T9SS.

A further subset of proteins is required for gliding in addition to the T9SS core proteins. These gliding-specific proteins are named GldABD and GldF-J and are predicted to localise to the cytoplasmic membrane (CM, GldAFG), or periplasmic side of the OM (GldDBHIJ). The OM-localised proteins are lipoproteins and one or more of these proteins are hypothesised to form the helical track that guides the movement of the adhesins (Braun, McBride, 2005)(McBride, Braun, 2004)(McBride et al., 2003)(Hunnicut, McBride, 2001)(Hunnicut, McBride, 2000b). The most important of these proteins is GldJ, as deletion of any one of the *gldABDFGHI* genes results in post-translational degradation of GldJ. GldJ, in turn, is homologous to and required for the stability of the T9SS-core protein, GldK. Detailed study of the exact role of these gliding proteins is therefore difficult as any null mutations will result in loss of T9SS function which will prevent adhesin secretion. Furthermore, a mutant of *gldJ* (*gldJ₅₄₈*) that encodes a 13

residue C-terminally truncated GldJ protein, GldJ₅₄₈, produces secreted but immobile adhesins. Thus, the *gldJ*₅₄₈ mutant supports an active secretion system but cannot power adhesin movement.

In the model organism *F. johnsoniae*, the major adhesin is SprB which is essential for colony spreading and gliding on PY2 agar and the most important adhesin for gliding on glass. *sprB* is encoded in an operon that comprises the genes *remF*, *remG*, *fjoh_0982*, *sprC*, *sprD*, *sprB*, and *sprF*. While *fjoh_0982* encodes a protein of unknown function that is not required for gliding, deletion of any one of the *sprCDBF* genes results in loss of gliding and colony spreading on PY2 agar (Nelson et al., 2008). Whilst mutation of *remF* or *remG* does not prevent PY2 agar colony spreading or gliding both of these genes have paralogs (*remH* and *remI*, respectively) and strains with mutations in both members of the pair (*remFH* or *remGI* strains) no longer glide on PY2 agar. Despite their importance for gliding, a mutant lacking all four genes (*remFHGI*) produce helically moving cell surface-localised SprB (Rhodes et al., 2011b).

SprF is an OM β -barrel protein from the PorP-family. It is required for the secretion of SprB onto the cell surface, whereas the other *sprB* operon genes are not (Rhodes et al., 2011a). The role of SprF in SprB secretion is not clear, but SprF likely acts as a shuttle protein similar to the role of PorV in the secretion of Type A CTD proteins (Lauber et al., 2018). Furthermore, secretion of SprB seems to require SprF in a stoichiometric relationship and SprB protects SprF from proteolysis by proteinase K supporting a model in which the two proteins exist in a complex after secretion of SprB (Kulkarni et al., 2019). There are 9 paralogs of *sprF* outside the *sprB* operon in the *F. johnsoniae* genome and

they are usually encoded next to type B CTD-encoding proteins such as SprB. SprF-like proteins are specific for their cognate Type B CTD domain-containing proteins, and SprF cannot be functionally replaced by one of its paralogs to support secretion of SprB (Kulkarni et al., 2019).

sprC encode a 483 residue periplasmic protein with limited similarity to SprB near the C-terminus end (28% identity over 239 amino acids) (Rhodes et al., 2011a). *sprD* encode a 1588 residue protein with a N-terminal ~ 300 residue OM β -barrel and a large coiled-coil domain predicted to be periplasmic (Rhodes et al., 2011a). Despite being phenotypically similar to a $\Delta sprB$ mutant, mutants lacking *sprC* or *sprD* produces secreted and helically moving SprB with similar abundance to that of the wild type (WT) (Rhodes et al., 2011a). Nevertheless, Kulkarni et al. (2019) reported that a mutant lacking both *sprCD* produced immobile and secreted SprB (Kulkarni et al., 2019).

Following the hypothesis put forth by Nakane et al. (2013), two major questions that remain unanswered so far are how SprB manages to distinguish leading from lagging cell pole during movement and how the cell control adhesion as a function of its direction of movement. In this chapter, I attempt to answer these questions by investigating the subcellular localisation and movement of proteins encoded by the *sprB* operon using the 3-dimensional (3D) single-particle tracking (SPT) methodology developed in chapter 3.

4.2 Results

4.2.1 Helical movement of SprB is independent of RemF, RemG, SprC, and SprD

sprB operon encoded proteins are essential for *F. johnsoniae* SprB-mediated colony spreading and gliding on PY2 agar and glass. However, apart from being essential, little is known about their mechanistic role. Therefore, I started my study of the *sprB* operon encoded proteins by assessing the domain architecture and compiling the available data presented in the literature into Figure 4.2.

RemF did not have any predicted domains except the Sec signal peptide. The Phyre2 structure prediction search did not retrieve any structural templates with high confidence, but the secondary structure was predicted with high confidence. RemG also did not have any predicted domains, but the Phyre2 structure prediction search found several templates with high confidence. The most confident template match (98.2 confidence score, 17% identity from residue 41-364) was the endo-xylogalacturonan hydrolase from *Aspergillus tubingensis*.

SprC possess two Ig-like fold domains (Figure 4.2 black domains). The Phyre2 structure prediction search retrieved several Ig-fold containing proteins that had high confidence scores. Most hits were human proteins, although the second-highest confidence (98.7) scoring protein template was the *Porphyromonas gingivalis* T9SS protein PorU, with 18% identity from residue 365-483 of the SprC C-terminal end to the C-terminal end of PorU (residues 1018-1156). Interestingly, this region is also similar to SprB. The similarity of

this region to SprB, noted by Rhodes et al. (2011a), were several repeats in the N-terminal half of SprB and not the Type B CTD domain. Thus, it seems that SprC contains a degenerate T9SS substrate CTD. To ensure that SprC was not secreted by this domain, I performed a Proteinase K shaving assay as described in 2.3.3. This assay indicates that SprC resides in the periplasm (Figure 4.1).

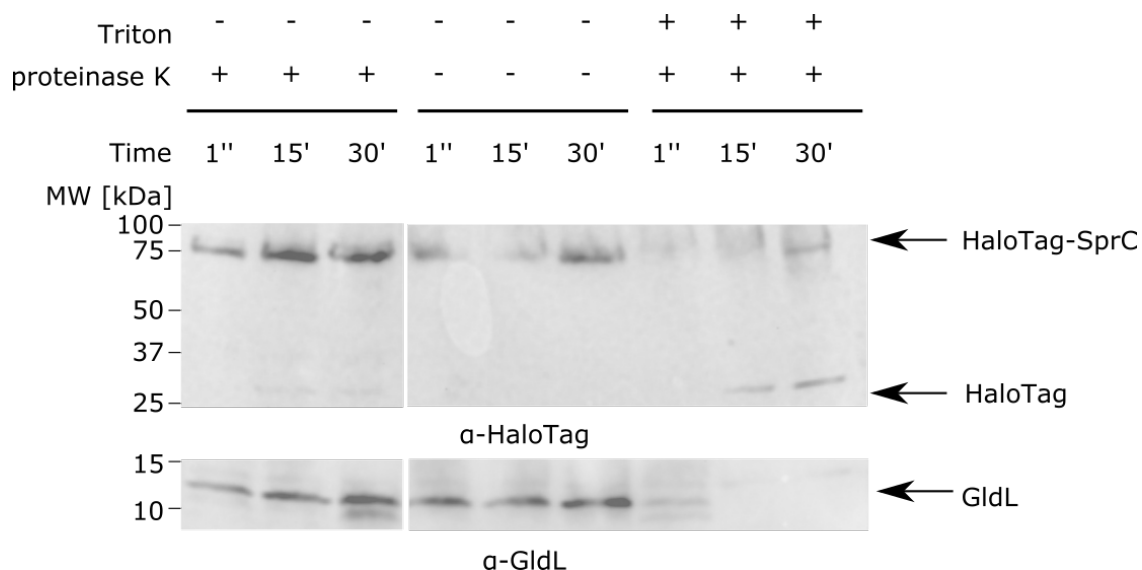


Figure 4.1: Proteinase K mediated proteolysis of SprC. A strain producing Halo-SprC was grown, treated with proteinase K, and prepared for immunoblot analysis as described in 2.3.3. The samples were split into 3 proteinase K (Sample), buffer (Control), or proteinase K and Triton (Triton). Immunoblots, first with α -HaloTag antibody (top blot) and thereafter α -GldL (bottom blot) to verify that proteins within the cells were only cleaved when Triton was added. The experiment was repeated 3 times.

SprD had a PorP/SprF-like (TIGR03519) domain predicted from residue 1-301 followed by a large number of coiled-coil domains predicted using the automatic sequence annotation tool provided by Uniprot and based on (Lupas et al., 1991). The region covered by coiled-coil domains is also a low-complexity and disordered region, thus it was not possible to

obtain a meaningful structure prediction of this region using Phyre2. Residues 1-309 of the SprD N-terminal domain were used to predict the structure using Phyre2 (Figure 4.2). The most confident hit was PorV (99.7) with 14 % identity from residue 44-308 of the query sequence, aligned with PorV residue 58-402. The SprD C-terminal residues 1154-1588 were used to predict the structure using Phyre2 (Figure 4.2). The C-terminal domain hit many predominantly helical proteins with different tertiary structures. Of the top hits were an intraflagellar transport protein from *Trypanosoma brucei*, DNA and protein binding proteins, contractile proteins and pyocin s5 from *Pseudomonas aeruginosa*. Because this domain can adopt so many different conformations with the coiled-coil structure it is difficult to judge what the function of this domain might be.

The SprB domain structure shows two large repeats shown on Figure 4.2 as blue bars (92% self identity) and purple bars (99% self identity), as well as several other smaller repeats such as the SprB repeat (Figure 4.2, red bars, Pfam ID PF13573) (Nelson et al., 2008). A T9SS Type B CTD (TIGR04131) is shown in yellow from residue 6414-6497 on Figure 4.2. A single repeat of the blue and purple, and the C-terminal domain, was used to predict their structure by Phyre2. The blue and purple repeats both had a large number of immunoglobulin-like domains and the best hit were carcinoembryonic antigen for both repeats (blue; 99.2 confidence, 13% identity over 88% of domain; purple, 98.4 confidence, 11% identity over 99% of the domain). Carcinoembryonic antigen proteins of the immunoglobulin superfamily are known for their role in cell-cell adhesion and are heavily glycosylated (Kuespert et al., 2006).

Unsurprisingly, SprF has a PorP-like domain (TIGR03519) predicted. Similar to the N-

terminal of SprD, the secondary structure prediction with Phyre2 identified PorV as the best template with 99.8 confidence and 16% identity from residue 17-295 (PorV 58-400). This is in accordance with the model wherein SprF operate similar to PorV in the secretion of SprB through the T9SS.

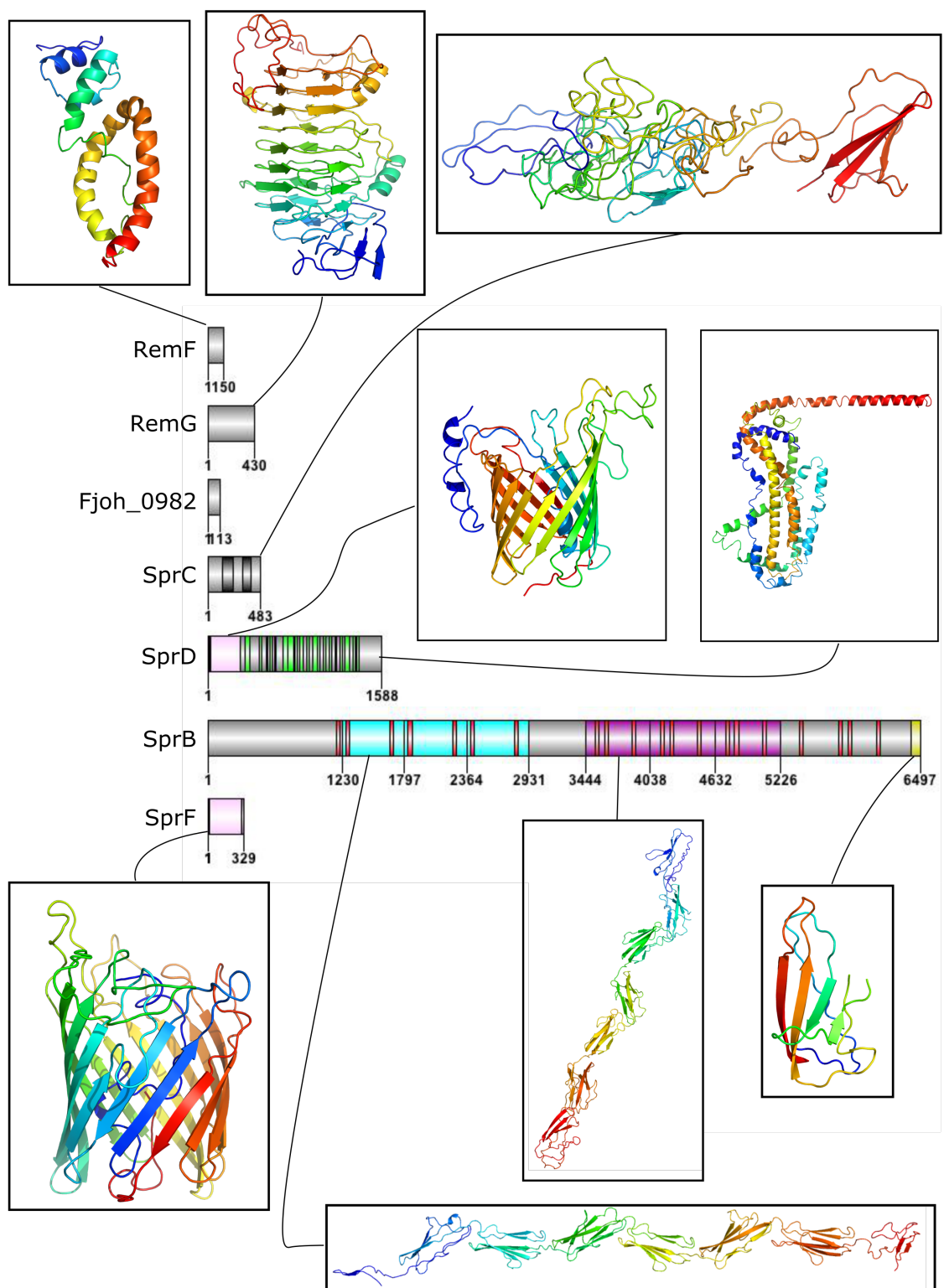


Figure 4.2: Predicted domains and structures of the *sprB* operon encoded proteins. Each *sprB* operon encoded protein is drawn to scale with domains drawn. Immunoglobulin (black, G3DSA:2.60.40.10), Coiled-Coil (green), PorP-like (pink, TIGR03519), and SprB repeats (red, PF13573; blue, 92% self identity; and purple 99% self identity) are shown. Sec signal peptides are not shown as a domain but all proteins have it. Whole proteins (RemF, RemG, SprC, SprF) or domains of larger proteins (SprD and SprB) had their structure predicted using Phyre2 with intensive mode (Kelley et al., 2015). Fjoh_0982 was not submitted to Phyre2. SprD structure predictions were done with the N-terminal domain (residues 1-309) and C-terminal domain (residues 1154-1588). SprB structure predictions were done for the repeats (residues 1230-1797 and 3444-4038) and C-terminal domain (residues 6414-6497). All structures are drawn as cartoons and coloured blue to red from the N- to C-terminus.

Kulkarni et al. (2019) reported that the *sprB* operon proteins SprC and SprD are required for helical movement of a 218 residue C-terminal SprB fragment fused at the N-terminus to superfolded green fluorescent protein (sfGFP-SprB_{CTD218}). Since individual *sprC* or *sprD* deletion mutants are able to produce helically moving SprB (Rhodes et al., 2011a) it suggests that SprC and SprD might have redundant functions. The amino acid sequence of SprC and SprD does not support this idea, as there are no common domains between the two proteins, and the structural predictions also suggest they adopt very different structures (Figure 4.2).

I decided to reassess the effect of each non-gliding *sprB* operon mutant on SprB helical movement by using the 3D SPT methodology described in Chapter 3. This methodology allowed me to identify potential alterations in the pattern of SprB movement, which could have been overlooked in previous studies. To do this, the Halo-SprB producing parental strain (*halo::sprB* Δ *porV*) was used as a background in which to study the individual *sprB* operon mutants. Since there are functionally redundant copies of *remF* and *remG*

in the genome of *F. johnsoniae* I generated the double mutants $\Delta remFH$ and $\Delta remGI$ to evaluate their role in gliding. I also assessed the double-mutant $\Delta sprCD$, reported to be deficient in SprB helical motion (Kulkarni et al., 2019).

The Halo-SprB fusion protein was stably expressed at similar levels in all the *sprB* operon mutant backgrounds (Figure 4.3, **a**). None of the mutant strains were capable of mediating colony spreading on PY2 agar plates (Figure 4.3 **b**). This was expected since it has been reported that all of these mutants prevent colony spreading when constructed in a WT background (Rhodes et al., 2011a)(Rhodes et al., 2011b)(Nelson et al., 2008). To understand the effect of these mutations on Halo-SprB movement, I performed 3D SPT. All mutants moved helically with no obvious deviations from the helical movement seen in the parental strain (Figure 4.3 **c-e**). This result was expected for all but the $\Delta sprCD$ mutant. The lack of helical SprB movement reported by Kulkarni et al. (2019) for a $\Delta sprCD$ mutant could be explained by a change in the proportion of helical molecules compared to the parental strain. I, therefore, quantified the frequency of helical molecules to understand if some mutants had a smaller proportion of helical molecules than the parental strain. To do this, tracks were classified as either helical or not using the random forest machine learning classification program (chapter 3).

Significant day-to-day variation was observed in all the strains and thus a substantial spread in the data can be seen (Figure 4.3 **f**). Furthermore, an inspection of the data distribution suggested that it was zero-inflated. Therefore, the data were modelled using a general additive model with a zero-inflated beta distribution and weighted by the total number of tracks. Using this model, pairwise comparisons of each mutant to the parental

strain were performed. The comparisons did show a significant ($p < 0.05$) reduction (Figure 4.3, **f**). Nonetheless, these results show that the *sprB* operon components are not required for the helical movement of SprB and so must have a different role(s) in SprB-mediated gliding.

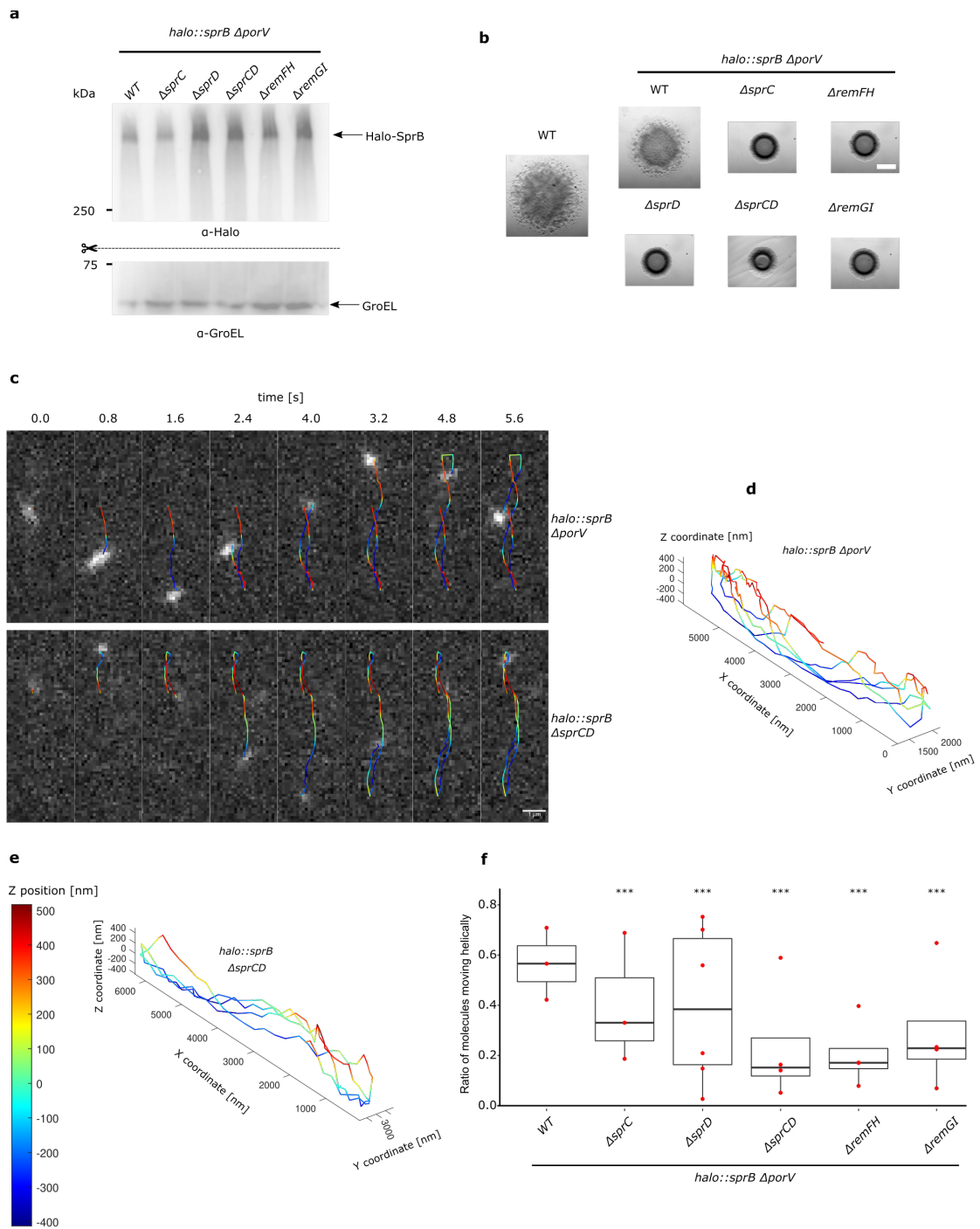


Figure 4.3: Strains with mutations in the *sprB* operon in a parental strain expressing Halo-SprB were grown as described in 2.3.2. (a) Whole cell immunoblots using the indicated antisera. GroEL is a loading control. The experiment was repeated 3 times. (b) Colony spreading on PY2 agar after 24 hours. The scalebar indicates 3 mm. WT, wild type cells not expressing Halo-SprB. The experiment was repeated 3 times. (c-f) Strains were grown, prepared, and imaged as described in 2.4.1. Cells were labelled with 0.01 nM JF646-Halo, mixed with SynaptoGreen[™] C4 membrane dye, and deposited on agar pads. The 3D SPT data was processed as described in 2.2. (c) Panels show every 10th frame of representative movies of the indicates strains. The track followed by the fluorescent SprB molecule is overlaid on the raw images coloured according to the position in the Z axis according to the key in e. The full tracks of the parental strain (d) and the $\Delta sprCD$ mutant (e) are shown in a 3D view. The proportion of helically moving fluorescent molecules was determined using the classification algorithm described in 2.4. (f) Boxplot showing the proportion of helically moving SprB molecules in a parental (406 tracks), $\Delta sprC$ (614 tracks), $\Delta sprD$ (1052 tracks), $\Delta sprCD$ (1326 tracks), $\Delta remFH$ (821 tracks), and $\Delta remGI$ (454 tracks) background. Each point represents the average propotion of helically moving molecules across all fields of view collected on that date. The data was modelled with a generalised additive model (GAM) using a zero-inflated beta distribution weighted by total number of tracks. Pairwise comparisons were made and corrected with the Bonferroni method. *** indicates significant difference ($p < 0.001$) between the parental and mutant strain.

4.2.2 Proteins of the *sprB* operon are required for SprB-mediated adhesion

Nakane et al. (2013) proposed a physical model for *F. johnsoniae* gliding motility in which a major assumption was that the gliding machinery could turn adhesion on and off during gliding. In this model, the ability of SprB to adhere only when moving from the leading to the lagging pole is necessary for successful net movement. Since the *sprB* operon proteins are required for SprB-mediated gliding, but not SprB movement (above), I hypothesised that they might instead be controlling the adhesive activity of SprB. To test this, I sought

to assess the effect of mutations in the *sprB* operon on SprB-mediated adhesion to glass tunnel slides. There are multiple adhesins encoded in the *F. johnsoniae* genome (McBride et al., 2009)(Shrivastava et al., 2012) potentially complicating assessment of the specific adhesive properties of SprB. However, the only other adhesins that have been experimentally shown to be required for attachment to glass slides are RemA and its paralogs (Shrivastava et al., 2012). These adhesins, but not SprB, require PorV for secretion and adhesion to the cell surface (Kharade, McBride, 2015). Thus, in a $\Delta porV$ genetic background adhesion of cells to glass should depend only on SprB and so this background was used in my adhesion assays.

To assess adhesion to glass, cell suspension of interest was first injected into tunnel slides and incubated for 5 min. Unbound cells were then washed away to leave only the cells that adhere to glass.

As an internal control, the test cells used in these experiments were mixed with the parental strain. All strains involved in the experiment produced a fusion protein with the HaloTag domain fused to SprB, so I used HaloTag labelling at saturating concentration of dye to stain the cells. The two types of strain were stained with different fluorescent dyes to allow the test and parental cells to be distinguished in fluorescence images. To establish the exact proportions of each cell type in the sample under analysis I also spotted the sample onto agar pads. This allowed me to correct the observed ratio of test to parental strains in tunnel slides for any differences in starting amounts of the two cell types in the sample. As a positive control, the adhesive capability of the parental strain was compared with itself. The parental strain is attached to glass slides in a 1:1 ratio with itself. As

a negative control, I compared the adhesive ability of the parental strain with a strain that produced only the last 448 C-terminal residues of SprB (Halo-SprB_{CTD448}) that are incapable of SprB-mediated gliding (Kulkarni et al., 2019). The *halo::sprB_{CTD448}* mutant strain was attenuated in glass adhesion compared to the parental strain. The mutants of the *sprB* operon shown above to be unnecessary for SprB movement were significantly ($p < 0.05$) affected in their ability to adhere to glass compared to the parental strain (Figure 4.4 **b**) and not significantly different from *halo::sprB_{CTD448}*.

A 50-residue C-terminal truncation of SprD has been shown to phenocopy the full *sprD* deletion in being unable to support colony spreading on PY2 agar plates (Rhodes et al., 2011a). To understand if this C-terminal truncation is enough to abolish SprB-mediated glass adhesion I made the mutation (*sprD₁₅₃₈*) in the parental strain and tested the adhesion as described above. The *sprD₁₅₃₈* mutant was defective in adhesion to glass to a degree similar to that of the Δ *sprD* mutant and was not significantly different from the *halo::sprB_{CTD448}* mutant (Figure 4.4, **c**).

The putative track protein GldJ is required for SprB secretion and movement. A 13-residue C-terminal truncation of GldJ has been shown to allow secretion but not the movement of SprB (Johnston et al., 2018). To understand if this truncation would also affect SprB-mediated adhesion, I made the *gldJ₅₄₈* mutation in the parental strain and assessed the adhesive capability as described above. Similar to the other mutants, the truncation of GldJ abolishes SprB-mediated adhesion to glass and was not significantly ($p > 0.05$) different from the *halo::sprB_{CTD448}* mutant.

From these results, I conclude that the *sprB* operon components known to be required

for SprB-mediated gliding are also required for normal levels of adhesion to glass. This suggests that the *sprB* operon encoded proteins are involved in controlling SprB adhesion and offers an explanation as to why mutants with deletions in the *sprB* operon genes are gliding-deficient. The results do not, however, provide any explanation as to how the *sprB* operon encoded proteins might influence SprB adhesion. One clue might be the GldJ protein, as a similarly low proportion of *gldJ*₅₄₈ mutant cells stuck to the glass in the experiment. Because this mutant produces cell surface-localised but immobile SprB proteins, it may be that the remaining *sprB* operon proteins exert their effect on SprB through GldJ.

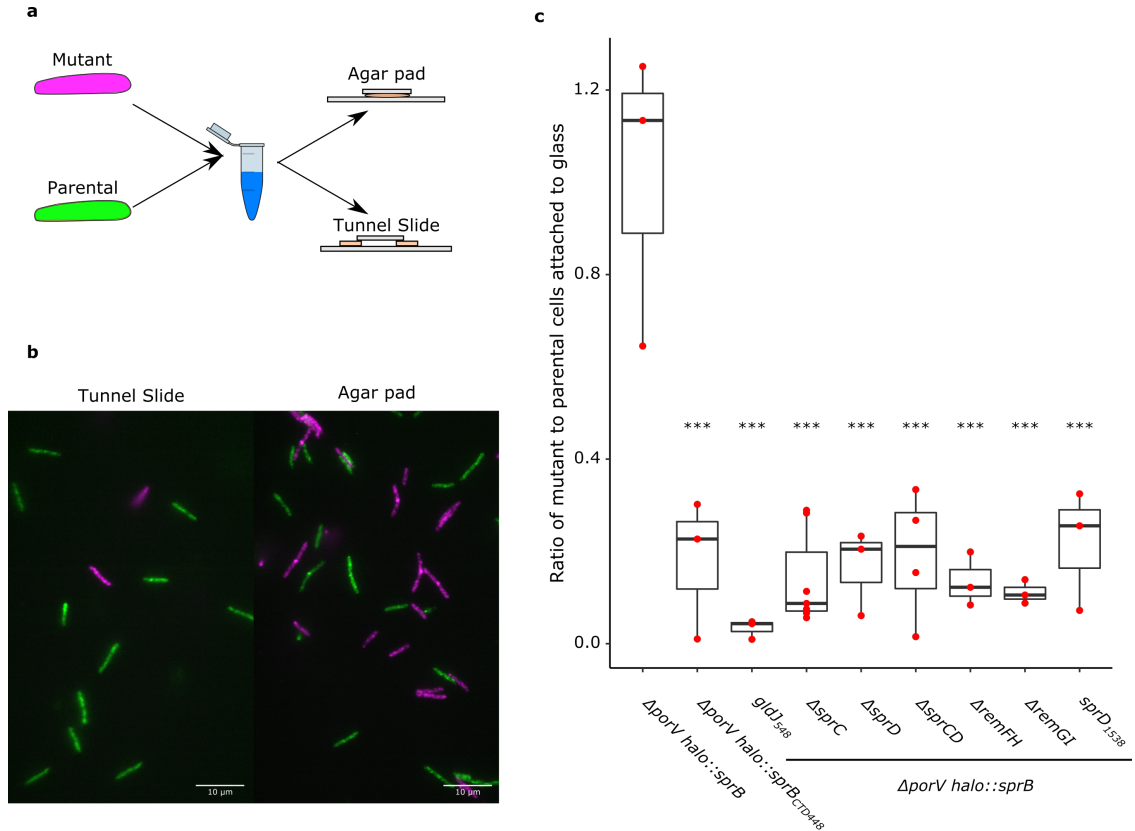


Figure 4.4: **(a)** Schematic illustration of the experimental strategy. The parental strain, *halo::sprB* $\Delta porV$ (green), was mixed 1:1 with a mutant strain (magenta). The mixture was analysed on agar pads to quantify the exact ratio of the two cell types and injected into tunnel slides and then washed to quantify cells with the ability to stick to the glass slide. **(b)** Representative microscopy images of strains grown as described in 2.4.1, labelled with 100 nM JF549-Halo or JF646-Halo dye and mixed as described in **a**. Green cells are the parental strain and magenta cells are a $\Delta sprC$ mutant in the parental background. Tunnel slide and agar slide images are from the same sample. **(c)** Ratio of mutant to parental cells. The number of cells in both the agar pads and tunnel slides were counted for a mix of the parental strain with itself (640 cells), a mutant expressing a truncated version of the Halo-SprB fusion (*halo::sprB*_{CTD448}, 444 cells) or one of the following mutants in the parental background: *gldJ*₅₄₈ (850 cells), $\Delta sprC$ (1504 cells), $\Delta sprD$ (489 cells), $\Delta sprCD$ (697 cells), $\Delta remFH$ (610 cells), $\Delta remGI$ (728 cells), *sprD*₁₅₃₈ (470 cells). The ratios of mutants relative to parental cells from the agar pads were used to adjust the ratios of mutants to parental cells in the tunnel slides. The average ratio across all fields of view for a given day was used to generate the boxplot. The data was modelled with a generalized least squares model and pairwise comparisons to the parental strain was performed using *a priori* contrasts. ***= $p < 0.001$.

4.2.3 Proteins of the *sprB* operon move in a helical track independently of SprB

sprB operon proteins are required for SprB-mediated adhesion to the substratum. However, they are not required for the helical movement of SprB. According to the current *F. johnsoniae* gliding model, the adhesion of SprB must be spatiotemporally regulated to ensure that adhesion only occurs when SprB is moving towards the lagging pole. Thus, *sprB* operon encoded proteins which modulate the adhesiveness of SprB must be able to exert their effect under these constraints. This could be achieved if the *sprB* operon proteins were also to move on the helical tracks, potentially as a complex with SprB. To test this idea, I decided to examine the movements of the *sprB* operon encoded proteins using the 3D SPT methodology described in Chapter 3.

In order to perform 3D SPT of the *sprB* operon proteins, HaloTag domains were appended either after the signal peptide (N-terminus of the mature protein) or at the C-terminus of the protein, without the use of linker regions. The RemF fusions were constructed and tested by Zoe Shipley as part of her Part II project. She also created the *remG::halo* conjugative plasmid.

The functionality of the RemF fusion proteins was assessed in a genetic background devoid of the RemF homologue RemH. Neither N- nor C-terminal HaloTag fusions to RemF were functional for gliding on agar plates. Immunoblots showed that only the N-terminal fusion protein was present at full length albeit with several cleavage products [Zoe Shipley, part II thesis, 2019]. These results are not surprising because RemF is a small protein (150

amino acids) that is unlikely to tolerate the addition of a large fusion protein domain. Inspection of SPT data collected from a $\Delta remH$ *halo::remF* strain did not show any fluorescent molecules moving in a helical pattern (data not shown). Thus, I did not pursue the study of RemF any further.

The functionality of RemG with HaloTag fused at the C-terminus was assessed in a genetic background devoid of the RemG homolog RemI. In this background, the RemG fusion protein was not able to mediate spreading suggesting that the fusion was not functional (Figure 4.5 **a**). However, I noticed that the same fusion inhibited spreading in a RemI-expressing background (Figure 4.5 **a**) suggesting that the non-functional RemG-Halo interacts with RemG and RemI interaction partners and may compete in a dominant-negative manner with the paralogous RemI protein. Immunoblotting with α -Halo antibodies shows that RemG-Halo is stable in both backgrounds. Similar results were observed for RemG fused to the N-terminus (data not shown) and only the C-terminal fusion was used for further study.

Strains producing a HaloTag fused to the N-terminus of SprC (Halo-SprC) formed spreading colonies, however the radius of the spreading colony was only about 50% of the WT (Figure 4.5 **a**). The N-terminal fusion was full-length and uncleaved (Figure 4.5 **b**). A C-terminal fusion was not made.

Cells producing HaloTag fused to the C-terminus of SprD (SprD-Halo) did not spread on PY2 plates (Figure 4.5 **a**). It is not surprising that addition of a HaloTag domain to the C-terminus of SprD disrupts function because the C-terminus of SprD is sensitive to small deletions as noted above (the *sprD*₁₅₃₈ mutant producing a 50 residue C-terminally

truncated SprD phenocopies a $\Delta sprD$ mutant). Immunoblotting of SprD-Halo revealed a full-length protein with no cleavage products (Figure 4.5 **b**).

3D SPT of the SprC, SprD, and RemG fusions detected helical protein movement resembling that of SprB (Figure 4.5 **c-d**). The proportion of helically moving proteins were lower for SprC ($\sim 4\%$), SprD ($\sim 3\%$) and RemG ($\sim 1\%$) than SprB ($\sim 55\%$, Figure 3.10). Nonetheless, proteins are highly unlikely to move helically if they are not engaged with the gliding machinery. The low percentage of helically moving molecules probably reflects the limited functionality of the fusion proteins. In addition, SprC were seen to more frequently stop in its tracks and stay immobile for periods before restarting movement. Such behaviour will also inflate the number of non-helical tracks classified by the machine learning algorithm.

SprB is thought to be connected to the periplasmically-located track proteins via the outer membrane protein SprF. I hypothesised that SprF might also mediate the interaction of the other helically moving SprB operon proteins with the track. To test this idea I investigated whether the other *sprB* operon proteins also depend on SprF for helical movement. I deleted *sprF* in each of the *halo::sprC*, *sprD::halo*, and *remG::halo* backgrounds and checked whether it had an effect on the proportion of helical molecules. No significant differences were observed between the *halo::sprC* mutant with or without *sprF* or the *remG::halo* mutant with or without *sprF*, in the $\Delta remI$ background (Figure 4.5 **d**). Significant differences were seen for the WT background when comparing RemG-Halo with and without *sprF*. Given there is no difference in the $\Delta remI$ background, the observed difference in the WT background is unlikely to be important. The *sprD::halo* mutant

with and without *sprF* where significantly different from each other ($p<0.001$). Regardless, *sprF* is not required for helical motion of SprC, SprD, or RemG as each molecule was observed to move helically in a $\Delta sprF$ background. Thus, it seems that the SprF-SprB and SprC-SprD-RemG protein groups act independently of each other with respect to helical movement.

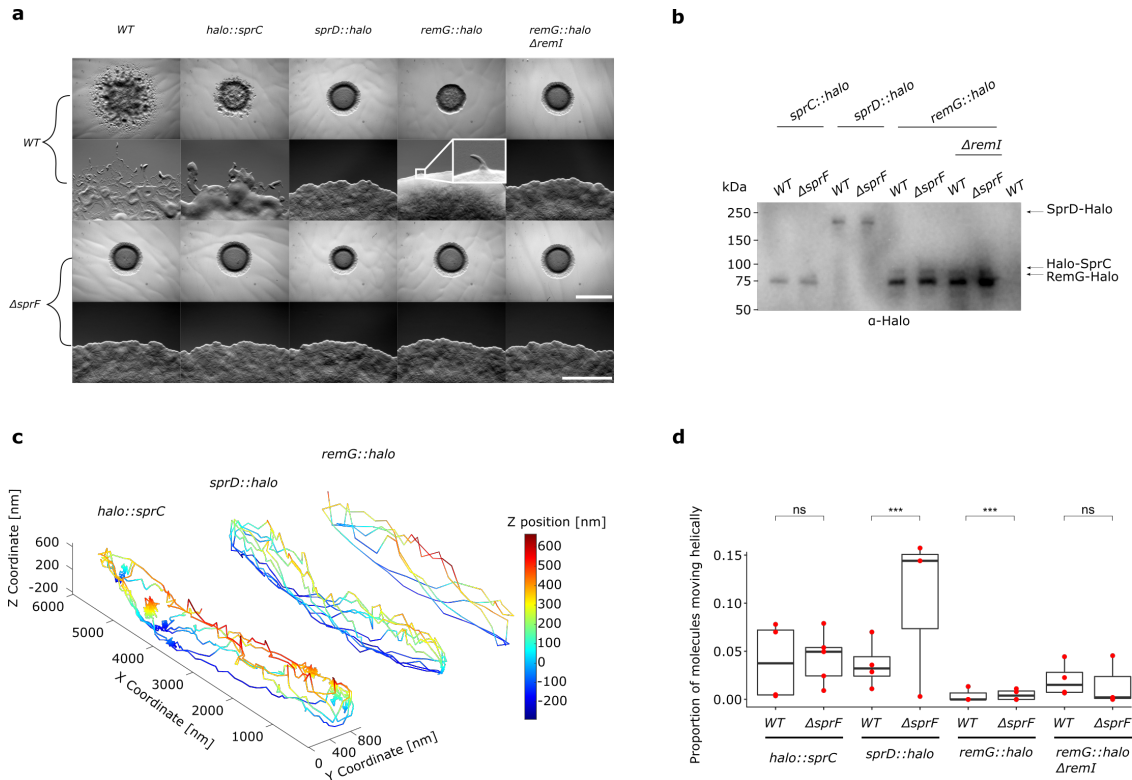


Figure 4.5: HaloTag fusion proteins from the *sprB* operon exhibit helical movement, independent of SprF. *halo::sprC*, *sprD::halo*, *remG::halo*, and $\Delta remI remG::halo$ mutant strains with or without a further $\Delta sprF$ mutation were grown as described in 2.3.2 and used for spotting PY2 plates (a) and collecting samples for immunoblot analysis (b). (a) The PY2 agar plates included a negative ($\Delta sprF$) and positive (WT) control for colony spreading. The PY2 agar plates were incubated for 24 hours before imaging. The white scale bar corresponds to 3 mm for whole-colony images and 0.5 mm for the magnified images. The experiment was repeated 3 times. (b) Immunoblot using α -HaloTag antibodies. The experiment was repeated 3 times. (c) The mutant strains used in a-b were grown and imaged as described in 2.4.1. The mutants were labelled with 0.05 nM (*halo::sprC*) or 0.1 nM (*sprD::halo* and $\Delta remI remG::halo$) JF646-Halo to obtain 1-2 fluorescent molecules per cell. The 3D SPT data were preprocessed as described in 2.2. Example single-particle tracking data for each of the three mutants were plotted with a line coloured by the position in Z and displayed in a 3D view. Each track is scaled to the same axis and colour-key. (d) Proportion of helically moving SprB were quantified in: a *halo::sprC* mutant with (1551 tracks) or without (1585 tracks) *sprF*, a *sprD::halo* with (1431 tracks) or without (290 tracks) *sprF*, a *remG::halo* with (564 tracks) or without (1540) *sprF*, and a $\Delta remI remG::halo$ mutant with (1728) or without (723) *sprF*. The data was modelled with a generalised additive model (GAM) using a zero-inflated beta distribution and weighting by total number of tracks. Pairwise comparisons were made as indicated and corrected with the Bonferroni method. ns indicates no significant difference ($p > 0.05$) between the parental and mutant strain. ***= $p < 0.001$

SprF and SprD are the most likely links to the helical track

RemG, SprC and SprD all move helically independent of SprF suggesting they interact with the gliding tracks in another way. Because the N-terminal domain of SprD contains a predicted SprF-like domain (Figure 4.2), it is possible that SprD interacts with the gliding track in a similar way to how SprF is predicted to interact with, and connect SprB to, the track. If this would be the case, SprD could act as the link between the remaining *sprB* operon proteins and the track.

To test this hypothesis, I investigated the ability of SprC and SprD to move helically, in

a background lacking *sprD* or *sprC*, respectively. The lack of *sprC* or *sprD* did not have any effect on the stability of the fusion proteins (Figure 4.6 **a-b**) and neither mutant were capable of forming spreading colonies on PY2 agar, as expected (Figure 4.6 **c**). Helical movement was not observed in either mutant (Figure 4.6 **d-e**). Since the proportion of SprD helical molecules is very low in the parental strain, deletion of *sprC* might reduce this to the point where the required number of tracks to resolve helical motion would be unrealistic given the experimental setup. Therefore, it is difficult to say whether *sprC* is a prerequisite for SprD-Halo helical movement or simply increases the functionality of the SprD-Halo fusion thereby increasing the likelihood of observing helical movement.

To further understand if the SprF-like N-terminal domain of SprD is responsible for the helical movement of *sprB* operon encoded proteins, I truncated *sprD* in the *halo::sprC* and *sprD::halo* backgrounds.

The smallest known truncation of SprD that phenocopies $\Delta sprD$ is a 50-residue C-terminal truncation of SprD. Therefore, I started by creating this mutant that expresses this SprD truncation with a HaloTag domain fused to the C-terminus (SprD₁₅₃₈-Halo). This fusion is stable (Figure 4.6 **a**) and unable to glide (Figure 4.6 **c**), as expected. However, when performing 3D SPT, clear helical trajectories could be seen (Figure 4.6 **e**). To ensure that the loss of gliding did not stem from disrupted SprC helical motion, I tested the ability of Halo-SprC to move helically in a *sprD*₁₅₃₈ mutant background. Halo-SprC was stable (Figure 4.6 **b**), and did not glide (Figure 4.6 **c**), as expected. Clear helical tracks could be seen for this strain as well (Figure 4.6 **d**). Thus, it seems the C-terminal end of SprD is important for gliding motility but not the helical movement of SprD and SprC. This result

does not exclude the possibility that SprD has multiple roles, and therefore I decided to investigate the N-terminal end of SprD as well.

Focusing on the role of the N-terminal domain of SprD in helical movement, I then created an N-terminal domain only truncation with an N-terminally fused HaloTag domain (SprD₃₀₉-Halo). This fusion protein was not stable and produced very low levels of un-cleaved protein (Figure 4.6 **a**). Therefore, I extended the N-terminal domain a further 324 residues towards the C-terminal domain (SprD₆₃₃-Halo) which resulted in an un-cleaved and stable fusion protein (Figure 4.6 **a**). As expected, the mutant producing SprD₃₀₉-Halo did not have any detectable helical molecules and the overall labelling was very low. However, the SprD₆₃₃-Halo mutant had helical molecule movement similar to the SprD-Halo producing mutant. This suggested that the N-terminal domain was sufficient to support the helical movement. Both mutants were unable to glide on agar as expected (Figure 4.6 **c**).

To understand if SprC relied on the N-terminal domain of SprD, the same N-terminal domain truncations were made (*sprD*₃₀₉ and *sprD*₆₃₃) in the *halo::sprC* background. Immunoblot analysis revealed that there was a slight (~15 kDa) difference between the two strains. Surprisingly, both the Δ *sprD* and *sprD*₃₀₉ mutants produced Halo-SprC that were ~15kDa larger than that of the parental strain. This suggests that Halo-SprC is processed in an SprD-dependent manner. In accordance with the immunoblot data, Halo-SprC moved helically only in the mutant producing SprD₆₃₃ and not in the one producing SprD₃₀₉ (Figure 4.6 **d**). The Halo-SprC molecules were frequently moving with a faster diffusion in the Δ *sprD* and *sprD*₃₀₉ mutants than the ones not moving helically in the

parental strain. Because SprC is a predicted periplasmic protein, loss of potential interaction with the track or SprD could lead to SprC freely diffusing in the periplasm. The random forest machine learning algorithm is likely worse at handling this type of movement, leading to more frequent misclassifications. I, therefore, inspected the tracks and found that all the tracks classified as helical were indeed not helical. An example of a wrongly classified track can be seen in Figure 4.6 **g**.

It is noteworthy that the N-terminal SprF-like domain of SprD is required and sufficient to mediate the helical movement of both SprC and SprD. These observations suggest that the C-terminal end of SprD is important for something other than helical movement since mutations of this part result in loss of SprB-mediated adhesion and colony spreading (above), but not the helical movement of SprC or SprD.

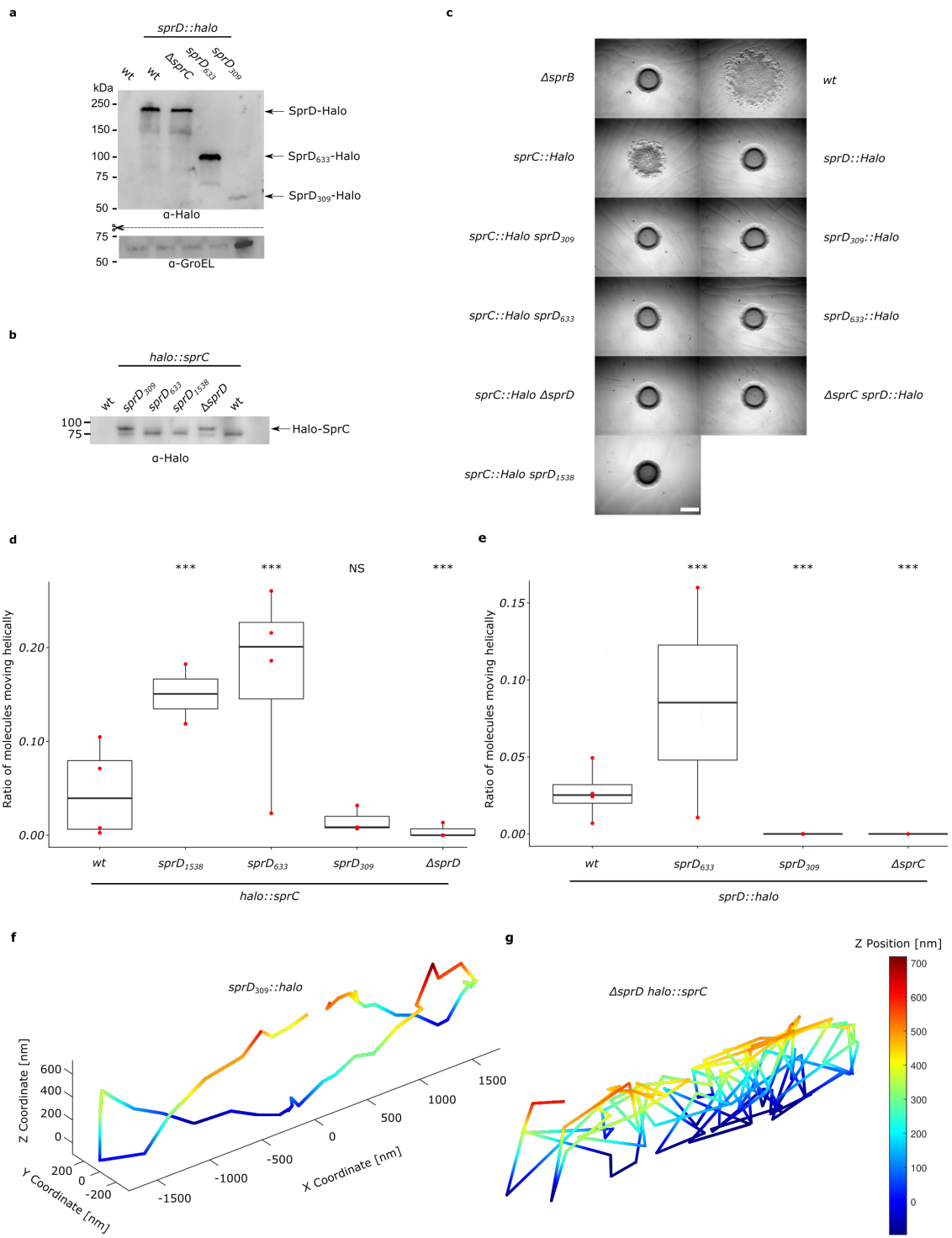


Figure 4.6: Mutations in *sprC* or *sprD* were introduced into strains producing either SprD-Halo or Halo-SprC fusion proteins, respectively. Parental and mutant strains were grown for immunoblot and PY2 agar colony spreading analysis as described in 2.3.2. **(a)** Immunoblot of strains producing fusions with HaloTag added to the C-terminus of SprD or SprD truncations. The SprD₃₀₉-Halo producing sample was loaded in 10-fold the amount. The immunoblot was probed with α -Halo antibody and α -GroEL antibody (as a loading control). The experiment was repeated 3 times. **(b)** Immunoblot of strains producing Halo-SprC. The experiment was repeated 3 times. **(c)** Images of colony spreading on PY2 agar plates after 24 hours growth. The scalebar indicates 3 mm. The experiment was repeated 3 times. **(d-e)** Strains used in **a-b** were grown, prepared, and imaged as described in 2.4.1. The strains were labelled with 0.05 nM JF646-Halo (*halo::sprC* strains or 0.1 nM JF646-Halo (*sprD::halo* strains) and mixed with SynptoGreen™ C-4 membrane dye. During imaging, a 1 on 3 off strobing frequency was used. The 3D SPT data were processed as described in 2.2 and each track was classified as either helical or not helical using the classification algorithm described in 2.4. **(d)** Boxplot of the proportion of helically moving Halo-SprC molecules in a WT (1551 tracks), *sprD*₆₃₃ (1070 tracks), *sprD*₃₀₉ (411 tracks), *sprD*₁₅₃₈ (1253 tracks), and Δ *sprD* (366 tracks) background. **(e)** Boxplot of the proportion of helically moving SprD-Halo (1431 tracks), SprD₆₃₃-Halo (1072 tracks), or SprD₃₀₉-Halo (35) in a WT background or SprD-Halo in a Δ *sprC* background (715 tracks) background. The *halo::sprC* mutant data is reproduced from Figure 4.5. The data was modelled using generalised additive models with a zero-inflated beta distribution and weighting by the total number of tracks. Pairwise comparisons were made to the parental strain in each boxplot and adjusted with the Bonferroni method. NS, not significant ($p>0.05$). ***, significant ($p<0.001$). **f-g** Example tracks from the *sprD*₃₀₉::*halo* (**f**) and Δ *sprD* *halo::sprC* (**g**).

4.2.4 SprC accumulates at the leading pole during active gliding

sprB operon encoded proteins move in a helix when cells are immobilised on agar pads. Because the agar pads trap cells between the glass slide and the agar, the cells are not able to glide and the *sprB* operon proteins are unable to mediate active gliding. This could be a problem because, during active motility, pressure-sensing is used to regulate

types of macromolecular motility machinery such as the Type IV pilus (T4P) or flagella (Dufrêne, Persat, 2020). Thus, potential *sprB* operon encoded protein behaviour could be missed with the experimental setup used so far. Therefore, I decided to label and observe mutants that produce *sprB* operon proteins fused to HaloTag during active gliding over a glass slide surface.

Because mutants with HaloTag fused to SprD or RemG are deficient in colony spreading on PY2 agar plates, I decided to use the Halo-SprC producing strain instead. The *halo::sprC* mutant is able to support colony spreading, albeit less so than the WT (Figure 4.6 c). Initially, the strains were examined for gliding in tunnel slides as previously described, but the cells rapidly consumed the available oxygen and became immobile at that point. Because of this, I decided to use flowcells coupled to a microfluidic pump that could supply fresh medium in a steady flow (described in detail in 2.4.1).

In gliding cells, a proportion of the fluorescent Halo-SprC formed a clear polar focus while, as observed on agarose immobilized cells, the remaining fluorescent Halo-SprC proteins moved helically both to and away from the leading pole (Figure 4.7).

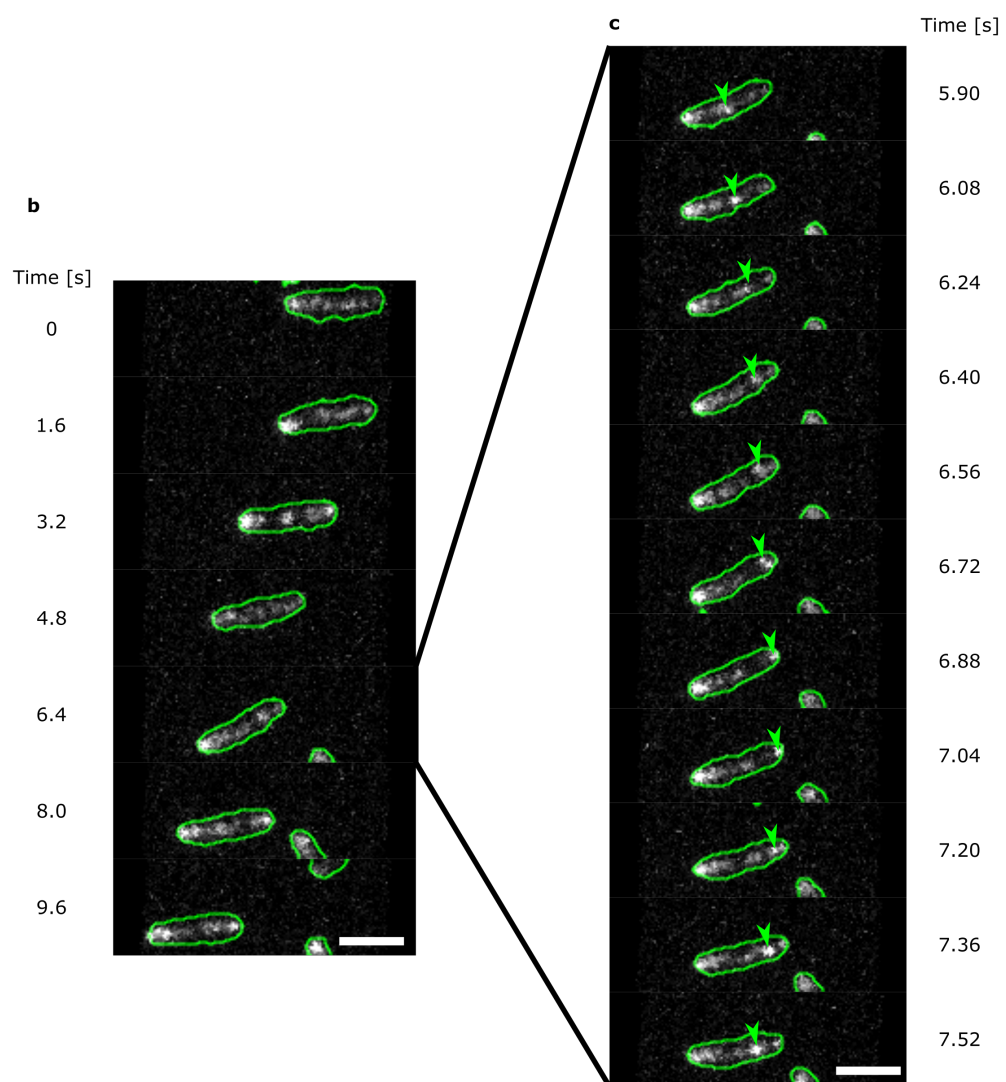
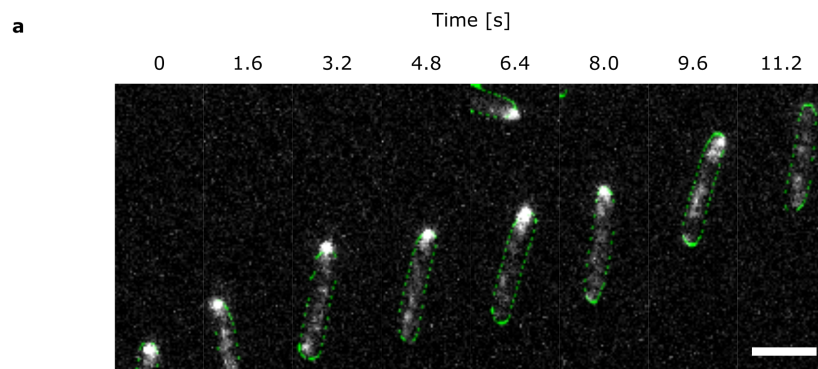


Figure 4.7: Gliding cells accumulates a portion of SprC at the leading pole. (**a-c**) A mutant expressing Halo-SprC was grown and prepared for imaging as described in 2.4.1, labelled with 100 nM JF646-Halo and imaged as described in 2.4.2 with 1 on/7 off strobing. (**a**) Panels from a movie of the mutant during active gliding with a polar focus. (**b-c**) Panels of a different cell from the same movie as **a**. Every 10th frame is shown in **b** and every frame from the indicated period is shown in **c**. Green arrowheads indicate a single SprC molecule moving in a helix during gliding. The scale bar indicates 1 μm .

The focus was consistently located at the leading pole of gliding cells. When observing non-moving cells on the same slide the fluorescence seemed equally distributed throughout the length of the cell. To analyse the polar localisation of Halo-SprC, I required a method of identifying the cell boundary in every video frame. Therefore, I added the cell membrane dye SynaptoGreenTM C-4 to the buffer flowing through the flowcell. The dye fluoresces when incorporated into the hydrophobic environment of a cell membrane, producing very clear cell outlines. By strobing the laser to limit photobleaching, long cell trajectories could be obtained wherein the cell outline could be identified from the channel containing the cell membrane dye signal. The coordinates of the cell boundary were then used to identify the cell area in the fluorescent channel. Using this technique, several cells with long time scale continuous movement and polar foci could be detected. From the tracking of the cells, the direction relative to the pole was calculated and compared to the intracellular fluorescent signal (Figure 4.8 **a-b**).

Occasionally cells that moved continuously gradually lost polar foci and the cell movement reduced (Figure 4.8 (**a-b**)). This phenomenon could be due to laser damage during extended exposure or it could be because the cells were preparing to reverse their direction.

Indeed, cells moving forward with net motion had occasional short reversals that seemed to become more frequent as a function of the declining intensity of the polar Halo-SprC focus.

To quantify the observed behaviour, the cells moving with a clear polar focus and net movement were selected. 47 cells were used to assess the correlation between subcellular fluorescence localisation and net movement. Every cell was rotated and normalised so that the most fluorescent pole corresponded to 1, mid-cell to 0, and the least fluorescent pole to -1. Normalised fluorescence showed clear polar foci at the +1 pole (Figure 4.8 c). However, in some cells, a less bright focus can be observed at the -1 pole. Speculatively, this could be in cases where the cell was preparing to reverse. The cells from the dataset used for polar fluorescence quantification were used to calculate the movement in each frame using a rolling 30-frame average. The histogram of the pooled frame-to-frame movement shows that the cells were biased in the movement towards the +1 pole (positive values) and a Gaussian fit ($R^2 = 0.966$) of the histogram reports the mean speed of cell movement to be $0.313 \frac{\mu m}{s} \pm 0.148 \frac{\mu m}{s}$ (SD). This is less than the reported speed of cell movement and SprB adhesin movement and likely reflects the occasional reversals, as well as eventually halting, of directed movement. Indeed, the right-handed side of the histogram lie between $1-2 \frac{\mu m}{s}$ suggesting the cells can achieve a maximum speed similar to the reported speeds. To test if SprB was also localising to the pole of gliding cells, I loaded a flowcell with both Halo-SprC and Halo-SprB producing strains, labelling them with different colour fluorophores. This experimental setup ensures that the conditions were the same for both strains. Like Halo-SprC, Halo-SprB were unmoving before I started to flow fresh buffer

through the flowcell. This is likely because of the lack of oxygen. After commencing the flow of fresh buffer, both Halo-SprB and Halo-SprC producing strains started to move and the proteins started to move around the cell bodies in the characteristic helical path within 2 min. A subset of cells also started to accumulate Halo-SprC at the cell pole at that point, however, this was not seen for Halo-SprB (4.8 **e-f**). This fits with the previous result that SprB is moving independently of the *sprB* operon proteins.

The 50 C-terminal residues of SprD are required for gliding on agar and SprB-mediated adhesion, but not for the helical movement of SprC. To investigate if the C-terminal SprD residues are required for polar localisation of SprC, a flowcell was loaded with Halo-SprC producing strains with or without a *sprD*₁₅₃₈ mutation. When the cells used up the oxygen, the patterns of immobile Halo-SprC were distributed throughout the cell length and were indistinguishable between the two strains. As fresh medium was flushed into the flowcell, both strains began propelling Halo-SprC around in a helix. However, the *sprD*₁₅₃₈ mutant did not accumulate Halo-SprC at the leading pole (4.8 **e-f**). As expected, the *sprD*₁₅₃₈ mutant also failed to support the net movement, more often staying within one cell length of its original position throughout the movie.

These results show that *F. johnsoniae* cells respond to their environment and SprC is recruited to the leading cell pole. The recruitment of SprC to the cell pole is dependent on active helical movement, as oxygen-depleted immobile SprC molecules were distributed throughout the cell. Although the exact signal is not known, I hypothesise that the active cell movement applies tension to the SprB filaments. SprB is not recruited to the pole during active gliding, however, and so the mechanism of polar accumulation is independent

of the localisation of SprB. Instead, polar accumulation depends on SprD and provides an explanation as to why the 50 residues C-terminal truncation SprD₁₅₃₈ protein is unable to support gliding on agar despite both SprC and SprB moving helically in this background.

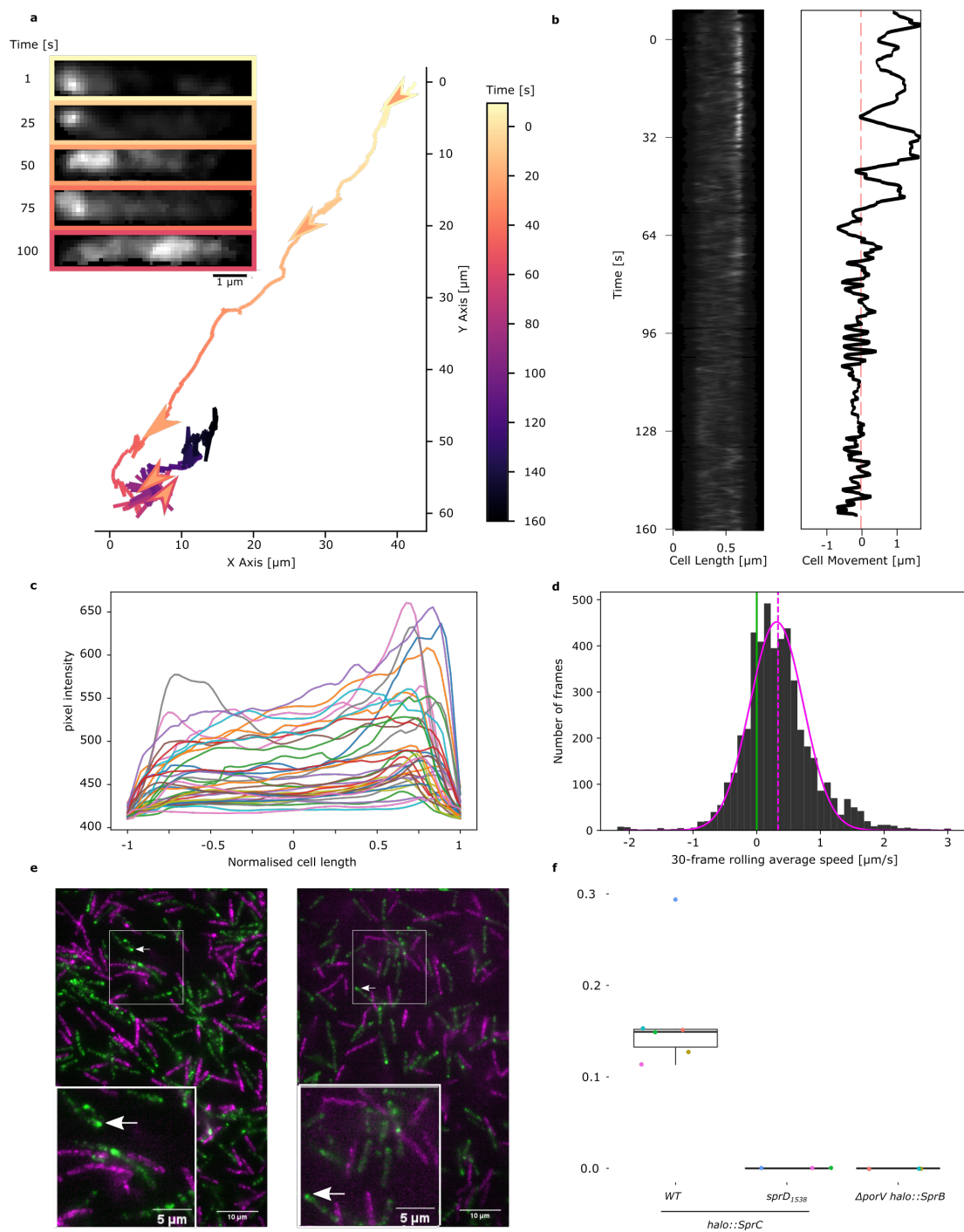


Figure 4.8: A *halo::sprC* mutant strain was grown and imaged as described in 2.4.1, labelled with 100nM JF646-Halo and SynaptoGreen™ C-4 before injecting into a flowcell. SHILO was used to collect every 8th or 25th frame, using 640 nm and 532 nm excitation. The cells were segmented using the signal in the channel with the SynaptoGreen™ dye, and the centre coordinate of the cells in each frame was used to track cell movement using the python package, trackpy (Allan et al., 2021). The cell outline coordinates were further used to crop out the cell area in the protein fluorescent signal channel in each frame. (a) An example cell trajectory with the Halo-SprC fluorescence is shown for the indicated timepoints. (b) The cell in a was used to calculate a kymograph (left) and the magnitude of movement (right) towards (+1) or away (-1) from the leading pole. 0 represents no net movement toward either pole. (c-d) 47 cells with clear net movement were selected. The cells were aligned relative to the leading pole. Positive X-axis values indicate the leading pole (c) and the movement towards this pole (d). (c) Internal fluorescence of each cell was drawn as a coloured line. (d) Histogram of pooled rolling average frame-to-frame displacement for all cells. (e) *halo::sprC* and either *halo::sprC sprD₁₅₃₈* or *halo-SprB ΔporV* strains were grown for microscopy as described in 2.4.1, labelled with either JF646-Halo or JF549-Halo, and injected into a flowcell. HILO microscopy was used to collect 100-frame videos with constant 20ms exposure, using both 532 nm and 640 nm lasers. (e) Single frames from the microscopy videos of *halo::sprC* (green) mixed with either *halo::sprC sprD₁₅₃₈* or *halo-SprB ΔporV* (magenta) are shown with a 120x120 pixel zoomed inset at the bottom left. A white arrow indicates a cell with a polar focus. (f) The number of all cells and the number of cells with a fluorescent polar focus were counted using the cell counter plugin in ImageJ. Boxplot with each data point representing the proportion of cells with a focus in a single field of view, and the colour denoting the biological replicate.

4.3 Discussion

Gliding motility in *F. johnsoniae* is achieved by helical movement of adhesins along the cell surface (Nakane et al., 2013). To accomplish net movement along the long axis of the cell, the adhesins, such as SprB, must be able to let go of the substratum during the period in which they move towards the leading pole. In *M. xanthus*, which uses an

evolutionarily unrelated mechanism of gliding motility, movement is also accomplished by helical movement of surface-exposed focal adhesion complexes (FAC) from leading to lagging pole (Mignot et al., 2007). The trans-envelope FAC is assembled at the leading pole where it attaches to the substratum and moves towards the lagging pole by using the force generated by the AglRQS proton motive force (PMF) harnessing proteins. Similar to *M. xanthus*, *F. johnsoniae* also encode PMF harnessing proteins (GldLM) that are necessary for powering gliding motility (Hennell James et al., 2021). AglRQS is homologous to the *E. coli* flagellar stator proteins MotAB which also generate PMF and forms a rotary motor (Deme et al., 2020). GldLM is not homologous to MotAB but forms a structurally similar complex and is also thought to rotate (Hennell James et al., 2021).

Both *M. xanthus* and *F. johnsoniae* use one or more proteins to produce a helical track that guides and/or interact with the adhesive complexes. *M. xanthus* use the cytoskeletal protein MreB to form a treadmilling helical cytosolic track at the base of the cytoplasmic membrane. The AglRQS proteins interact with MreB and are guided by the topology of the MreB filaments. By contrast, *F. johnsoniae* relies on periplasmic proteins to form a helical track. The most likely candidate for the helical track is the lipoprotein GldJ. GldJ is thought to form a helical track at the base of the OM and interact with the GldJ-homologous OM T9SS lipoprotein GldK.

It is noteworthy, that the speed of gliding in *M. xanthus* is much slower ($\sim 4 \frac{\mu m}{min}$) than that of *F. johnsoniae* ($\sim 2 \frac{\mu m}{s}$). One reason for this could be that the track of *M. xanthus* is located in the cytoplasm and therefore the challenge of moving the FAC through large obstacles in the periplasm such as the cell wall would slow it down. In accordance with

this hypothesis, AglR and MreB both move with speeds similar to SprB ($\sim 2 \frac{\mu m}{s}$) but has a sub-population that moves slower. AglR was reported to slow down near the leading pole, suggesting it engages with the FAC and starts moving slowly after forming the transenvelope complex (Nan et al., 2013)(Fu et al., 2018). Thus the FAC seem to comprise a speed limitation and I hypothesise that the fast movement observed for AglRQS can be attributed to the fact that they do not cross the cell wall.

Another reason that *M. xanthus* gliding is slower than *F. johnsoniae* can be due to the method of attachment/detachment. *M. xanthus* uses an adhesin that is thought to be secreted by the gliding machine at the leading pole and proteolysed at the lagging pole (Islam et al., 2020). This requires the adhesins to be continuously secreted and proteolysed for each run from the leading to the lagging pole. SprB in *F. johnsoniae* moves continuously between the two poles, and thus avoids this challenge. By doing so, however, *F. johnsoniae* must use another mechanism for controlling attachment/detachment. In this chapter, I present evidence that the *sprB* operon proteins are required for SprB-mediated attachment to glass. This finding suggests that the *sprB* operon proteins can control the adhesive state of SprB. The control has to be very fast, however, since SprB moves at $\sim 2 \frac{\mu m}{s}$ around the cell. To comply with this requirement, one solution would be for the *sprB* operon proteins to be in direct or bridged contact with SprB and thereby induce conformational changes that are far quicker than e.g. a diffusion-dependent response. Diffusion dependent responses are a limiting factor for generating fast responses in e.g. flagellar systems (the typical response time is $\sim 200ms$ (Segall et al., 1982)) and macromolecular chemotaxis complexes are often recruited close to the pole in polarly-flagellated

bacteria e.g. *Vibrio species* (Ringgaard et al., 2011). However, being in contact with a helically moving protein poses the obvious difficulty of following the protein around the cell. Remarkably, the *sprB* operon proteins SprC, SprD, and RemG can move in a helix reminiscent of the motion observed for SprB itself, despite being predicted periplasmic or OM proteins. This finding supports a model in which the *sprB* operon proteins interact with SprB to control adhesion, although the exact mechanism by which they affect SprB is still unknown. SprF is hypothesised to carry SprB around the cell, bridging the OM and allowing SprB to connect to the track formed on the periplasmic leaflet of the OM. It is interesting that the SprF-like N-terminal domain of SprD is required for the helical movement of the *sprB* operon proteins as well as this suggests that it may interact with the track in a similar way to SprF. Likewise, it could be that the similarity of the N-terminal end of SprD and SprF is a requirement for the mechanism by which SprB can interact with the *sprB* operon proteins. It is not possible, however, for SprD to support secretion of SprB in the absence of SprF (Rhodes et al., 2011a), so there is no direct evidence that SprB can interact with the SprF-like domain of SprD.

In addition to regulating adhesion over a short timescale, the regulation of SprB adhesion must be informed by the intracellular position, highlighting the need for gliding cells to discern and regulate which pole is leading and lagging. To discern the poles, *M. xanthus* uses a GTPase system in which the polar protein MglA indicates the leading (GTP state) or lagging (GDP state) pole. At the leading pole, MglA-GTP binds the track, stimulates motor assembly, and initiates the formation of the FAC. MglA-GTP moves with the FAC as it traverses the cell to the lagging pole. At the lagging pole, MglA-GTP is

dephosphorylated and can no longer bind the track or FAC. MglA-GDP is soluble in the cytoplasm and diffuses back to the leading pole where it is phosphorylated and can begin a new cycle. No such GTPase has been identified for *F. johnsoniae*, suggesting that *F. johnsoniae* uses a different mechanism to discern between the leading and lagging pole. Until now, there has been no evidence of asymmetry within the gliding motility system of *F. johnsoniae*. In this chapter, I present microscopy data that shows a polar accumulation of SprC at the leading pole in gliding cells. This polar accumulation was not observed for SprB, supporting a model in which the SprB-adhesion controlling proteins can move independently of SprB. Indeed, the C-terminal 50 residues of SprD are required for this polar accumulation of SprC while it is not required for the helical movement of SprC. Thus, a likely explanation is that *F. johnsoniae* has polar determining proteins that can interact with the *sprB* operon proteins to immobilise them and thereby bias the local concentration of adhesive control proteins to achieve net movement.

The *sprB* operon encoded proteins are important for SprB-mediated adhesion and both SprC and SprB follow the helical track during movement, independently of one another. Therefore, I hypothesise that the track is the connecting link between SprB and the remaining operon encoded proteins. In support of this, a truncation of GldJ (GldJ₅₄₈) also led to reduced SprB-mediated adhesion similar to the levels observed for the *sprB* operon mutants.

GldJ accumulates at mid-cell during cell division and can be found at higher concentrations at the pole in some cells Braun, McBride (2005). This polar distribution is reminiscent of the polar localisation of SprC in gliding cells and suggests that GldJ might

also respond to, or act as, a polar signal. However, since SprB depends on GldJ as well and does not accumulate at the pole of gliding cells, the more likely hypothesis seems to be that there are components required for polar accumulation. SprD contains a long C-terminal coiled-coil domain with some structural homology to contractile proteins. The extreme 50 residues of this domain are required for polar accumulation of SprC and it could potentially reach down and interact with a cytoplasmic polar anchor. Cytoplasmic polar anchors are known to recruit a broad variety of cell machinery in distantly related species such as HubP in *Vibrio cholerae* (Yamaichi et al., 2012), PodJ in *Caulobacter crescentus* (Viollier et al., 2002), and DivIVA in *Bacillus subtilis* (Thomaides et al., 2001). Gliding motility in *M. xanthus* and *F. johnsoniae* could be a result of convergent evolution. Where *M. xanthus* evolved a slower system, likely because of the cell-wall limitation, *F. johnsoniae* evolved the potentially fastest possible system. However, several questions still remain. *F. johnsoniae* cells are able to move toward a favourable milieu but do not have any homologs of the canonical chemotaxis proteins that are common amongst most motility systems including *M. xanthus* gliding motility. In *M. xanthus*, nucleoid-associated receptors signal the reversal of leading and lagging pole by activating response regulator proteins that diffuse to, and bind, cytoplasmic polar determinants. This signalling cascade results in a change in the direction of movement. *F. johnsoniae* does not have any cytoplasmic proteins that have been identified as important for gliding apart from the ABC-transporter whose function is required for GldJ stability (Johnston et al., 2018). Thus, it seems that the absence of cytoplasmic gliding motility proteins might have taken *F. johnsoniae* down a different evolutionary path. Indeed, the observation that SprC

accumulates only at the leading pole is the first example of an asymmetrically distributed gliding motility protein to date. This protein likely resides near the OM, attached to GldJ, and would therefore be unable to interact with any cytoplasmic signalling molecule directly.

In summary, *F. johnsoniae* gliding is controlled by modulating the adhesion of SprB via RemF, RemG, SprC, and SprD. RemG, SprC, SprD and likely also RemF, move along the helical track and are hypothesised to form transient interactions with an SprF-SprF complex to engage adhesion, forming a FAC. Polar accumulation of SprC and possible other *sprB* operon encoded proteins, ensure directional gliding by indicating the cellular position where SprB must adhere and FACs assemble. Upon travelling from leading to the lagging pole, a disassembly signal similar is thought to disengage the two subcomplexes, releasing SprB adhesion and allowing both the SprB-SprF and SprC+others to move helically back to the leading pole separately.

Chapter 5

Further investigation of the *sprB* operon

5.1 Introduction

Proteins encoded by the *sprB* operon are required for gliding motility in *F. johnsoniae*. However several of these proteins are neither required for secretion of the adhesin, SprB, nor for its movement. Instead, they seem to control the attachment/detachment of SprB. The exact mechanism by which the *sprB* operon proteins exert this control is not known, but at least one of the proteins, SprC, has been shown to specifically localise to the leading pole during active cell translocation (Chapter 4). It is thus hypothesised that SprC, and possibly other *sprB* operon proteins, regulate the adhesin to attach/detach at the right time. This regulation must occur over very short periods due to the fast velocity of SprB.

A possible model is that the *sprB* operon proteins form a transient complex with the adhesin that activates attachment for the period it takes the adhesin to move from the leading to the lagging pole.

In other motility systems, motility proteins are actively recruited to the poles of the two daughter cells during symmetric cell division. For example, in the polarly flagellated bacterium *V. cholera* an active process utilising ParA-like proteins to ensure that the chemotaxis proteins are distributed near both cell poles in a cell-cycle dependent manner) (Ringgaard et al., 2011). Kempf, McBride (2000) noted that *F. johnsoniae* stopped moving for 1 to 5 min as cells divided. Using immune electron microscopy, Braun, McBride (2005) observed enrichment of the proposed track protein GldJ at the mid-cell in dividing cells, suggesting that extensive track-remodelling could be occurring at these sites.

If the putative track formed by GldJ is the connecting link between the energy provided by the T9SS motor complex and the helically moving adhesins, then it must be able to interact with both. I have not been able to add any tags larger than a twin-strep tag without severely disrupting the function of GldJ. This makes sense because there would be little space for additional protein domains in such a tightly organised and interconnected environment. In this chapter, I will investigate if GldJ interacts with both the T9SS and the moving adhesins.

The ability to control attachment/detachment of adhesins represents a fundamental property of the gliding motility apparatus in *Bacteroidetes* and should therefore be present in all gliding species. There has been limited bioinformatics work done on the conservation of *sprB* and *sprB*-like operons in *Bacteroidetes*; in this chapter, I will therefore investigate

the distribution of *sprB*-like operons within *Bacteroidetes* with particular emphasis on the conservation of genes I have shown here to be involved in modulating adhesin attachment to the substratum.

5.2 Results

5.2.1 Motility proteins are recruited to the mid-cell during cell division

When cells with *sprB* operon proteins fused to the HaloTag domain (Halo-SprC, Halo-SprB, SprD-Halo) were imaged over long (~ 1 -2 hours) time periods, all cells in the population continued to have similar levels of fluorescent labelling even though cell division occur over this time period. Since no new proteins could be labelled after imaging commenced, this suggests that *F. johnsoniae* must be able to distribute pre-existing fluorescent proteins equally between daughter cells. A fluorescent focus was occasionally observed to form at mid-cell in dividing cells. This behaviour was transient and likely reflects a cellular strategy to split motility proteins equally between daughter cells. I decided to quantify this behaviour and revisited a dataset used for determining the SprB-mediated attachment to glass slides presented in Figure 4.4. In this experiment, I counted cells with fluorescently labelled SprB that had a mutation in one or more of the motility proteins. Each strain had been imaged on agar pads mixed with the parental strain. I counted the frequency of the mid-cell foci relative to the parental strain. This ratio was roughly equal between the

mutant and parental strain for all *sprB* operon mutants that affect SprB adhesions (Figure 5.1) suggesting that none of the products of these genes (SprC, SprD, RemF, RemG, RemI, RemH) is required for the accumulation of the adhesin to the division septum. By contrast, no mid-cell foci were observed in a *gldJ₅₄₈* mutant. The *GldJ₅₄₈* mutant is known to secrete but not propel SprB adhesins (Johnston et al., 2018). Suggesting that sorting of SprB to the cell division septum requires active movement of SprB along the gliding track.

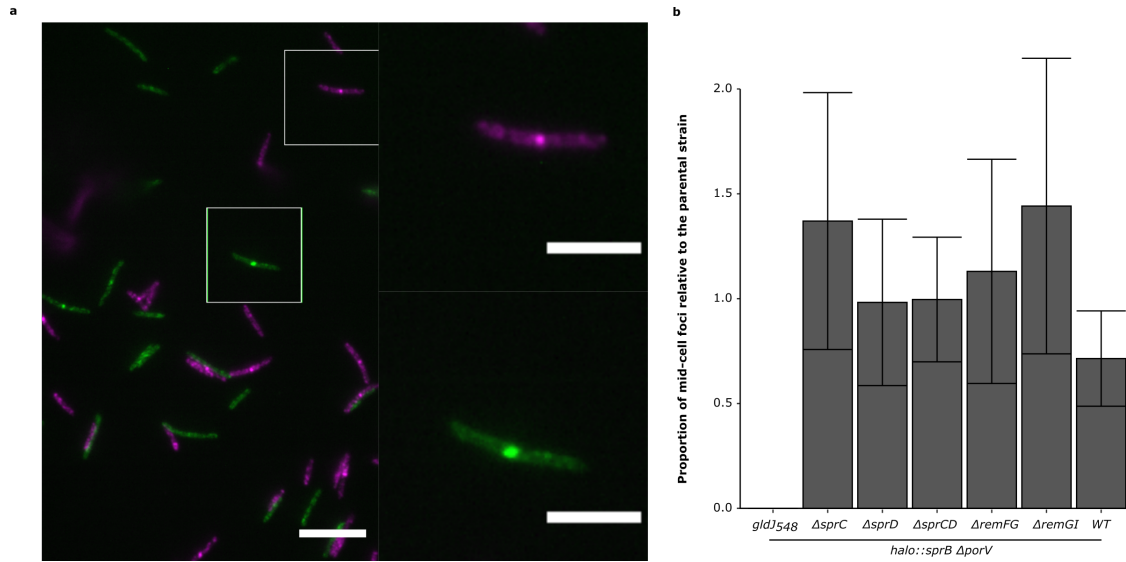


Figure 5.1: GldJ is required for accumulation of SprB at mid-cell during cell division. Data from Figure 4.4 were used to count the total number of cells as well as the number of cells with a fluorescent focus at midcell. **(a)** Example image of a field of view (left) and 120x120 pixel zooms of mutant ($\Delta porV \Delta sprC halo::sprB$, top right) and parental ($\Delta porV halo::sprB$, bottom right) cells with a midcell focus. The scalebar on the field of view is 10 μm and the scalebars on the zoomed images are 5 μm . **(b)** Boxplot representing the ratio of mutant to parental frequencies of midcell accumulated SprB. The number of foci per cell for both the mutant and parental strain were counted in each *gldJ*₅₄₈ (2870 cells), $\Delta sprC$ (2118 cells), $\Delta sprD$ (3429 cells), $\Delta sprCD$ (3116 cells), $\Delta remFH$ (2656 cells), $\Delta remGI$ (2141 cells), and parental (2622 cells) dataset. The focus per cell frequency for mutant and parental strain was used to calculate the relative ratio between them. 1-10% of the population had SprB accumulated at midcell in all strains except *gldJ*₅₄₈ in which no foci were observed.

5.2.2 Overproduction of a SprD C-terminal fragment causes aggregation of *sprB* operon proteins

It is hypothesised that SprC and SprB interact to initiate the attachment of SprB to the substratum. This interaction most likely takes place indirectly via other proteins since SprC and SprB are on opposite sides of the OM. An obvious candidate for a linking protein is the OM-spanning protein SprD which I have shown to be required for the movement of SprC on the gliding tracks. SprC polar accumulation in gliding cells depend on the SprD C-terminal 50 residues (Figure 4.8). The SprD C-terminus is also required for SprB-mediated attachment to glass (Figure 4.4), however, so it is not clear if it is a prerequisite or a requirement for polar accumulation of SprC. For example, the SprD C-terminus could be required as a polar recruiting agent of SprC or be required for SprB-mediated adhesion through an unknown mechanism that could be a prerequisite for SprC polar accumulation. To understand if the SprD C-terminal fragment is involved in bridging of SprC and SprB

function, I decided to see what would happen to SprC or SprB when this fragment was overproduced. To do this, I designed a plasmid that constitutively overproduces the fragment (*sprD*_{1154–1588}) from a *F. johnsoniae ompA* promoter (*pCP-sprD*_{1154–1588}).

*pCP-sprD*_{1154–1588} was transformed into mutant strains that produce either Halo-SprC or Halo-SprB. Both strains formed 1-2 bright foci per cell containing most of the labelled protein in the cells (Table 5.2.2). The foci appeared predominately near the cell poles but also throughout the cell body (Figure 5.2).

In this experiment, the mutant cells still produced the native SprD protein along with the ectopically produced SprD C-terminal fragment. To examine whether the formation of the SprB and SprC clusters depends on the native SprD protein, I deleted *sprD* in both the *halo::sprC* and *halo::sprB* strains carrying *pCP-sprD*_{1154–1588}. Overproduction of SprD_{1154–1588} in these strains resulted in a similar accumulation of fluorescently labelled protein to the ones with native SprD present (Table 5.2.2). Thus, the native SprD protein is not important for the accumulation of SprC or SprB in the SprD C-terminal fragment induced foci.

sprC might also have an influence on SprB behaviour in this experiment. Therefore, I deleted *sprCD* in the *halo::sprB pCP-sprD*_{1154–1588} strain. Halo-SprB still accumulated in immobile foci as a result of SprD C-terminal fragment overproduction, showing that neither native SprD nor native SprC is important for SprB aggregation under these experimental conditions.

Because the aggregation of SprC and SprB in the respective strains are phenotypically similar, I decided to investigate if the two proteins colocalised in the same clusters. To

do this, the HaloTag on SprC was replaced with a SNAP-tag. SNAP-tag is a domain that, like HaloTag, can be covalently labelled with synthetic dyes carrying a small ligand. No working antibody could be identified, so I was unable to verify the stability of this fusion. However, helical traces of fluorescent spots were seen similar to the Halo-SprC fusion, suggesting that the SNAP-fusion is functional (data not shown). In support of this, the *halo::sprB snap::sprC ΔporV* mutant spread approximately as well as the *halo::sprC* strain (Figure 5.2, **a**). Using this double-tagged mutant, I was able to label Halo-SprB with JF549-Halo and SNAP-SprC with JF669-SNAP allowing simultaneous observation of both proteins in separate channels on the microscope. To understand if the SprD C-terminal fragment induced SprC and SprB clusters were colocalised, I transformed *pCP-sprD_{1154–1588}* into the *snap::sprC ΔporV halo::sprB* mutant. Overproduction of the SprD_{1154–1588} fragment resulted in formation of colocalised bright foci of fluorescently labelled SNAP-SprC and Halo-SprB (Figure 5.2). Thus, the SprD C-terminal fragment is capable of inducing aggregates consisting of both SprC and SprB.

GldJ likely forms the track that all helical proteins follow. Since the C-terminal fragment of SprD alone was sufficient to cause SprC and SprB to aggregate together, one possibility is that the C-terminal fragment of SprD interacts with GldJ and thereby affects both SprC and SprB. I first decided to test if Halo-SprB aggregation was dependent on GldJ since direct interaction of extracellular-facing SprB is unlikely to interact directly with the overproduced SprD C-terminal fragment in the periplasm. Because a *ΔgldJ* mutant is unable to secrete SprB, I decided to use *gldJ₅₄₈* since this mutant is able to secrete but not propel SprB (Johnston et al., 2018). This mutant is also deficient in SprB-mediated

adhesion (Figure 4.4). Halo-SprB did not accumulate in this background, suggesting that the C-terminal fragment of SprD exerts its effect on SprB through GldJ.

SprC is located in the periplasm, and could possibly interact directly with SprD in addition to, or instead of, interacting through GldJ. To test if SprC was affected by mutations in *gldJ*, $\Delta gldJ$ or *gldJ*₅₄₈ mutations were made in the Halo-SprC producing strain carrying *pCP-sprD*_{1154–1588}. Both $\Delta gldJ$ *halo::sprC* and *gldJ*₅₄₈ *halo::sprC* strains carrying *pCP-sprD*_{1154–1588} displayed Halo-SprC foci similar to the ones reported above for the parental strain (Figure 5.2 and Table 5.2.2). Thus, it seems that SprC does not require GldJ for SprD C-terminal fragment induced aggregation. To further test if the core components of the T9SS could have an effect on the aggregation, I deleted *gldKLMNO* in the Halo-SprC producing strain carrying *pCP-sprD*_{1154–1588}. Similar to the other strains, this also had foci of Halo-SprC. In summary, the C-terminal fragment of SprD require GldJ to aggregate SprB, whereas GldJ and the T9SS are dispensible for SprC aggregation (Figure 5.2 and Table 5.2.2).

SprC aggregate in all backgrounds tested. This could either be because there is a true interaction between SprC and the C-terminal fragment of SprD or it could be due to random aggregate formation induced by the SprD C-terminal fragment. If the SprD C-terminal fragment itself could aggregate as a result of its overproduction, this could lead to the capture of random periplasmic proteins. SprB did not aggregate when *gldJ* were truncated (*gldJ*₅₄₈). This could be because GldJ is the sole link between SprB and the periplasmic proteins, thus the truncation resulted in SprB 'falling off the track'.

Nonetheless, the result suggests that GldJ serves as a link between the *sprB* operon

proteins and SprB. Furthermore, there may be an interaction of the SprD C-terminal domain with either GldJ or SprC although this requires further investigation.

Table 5.1: Summary of results from overproduction of SprD_{1154–1588} on SprB and SprC cluster formation. +, presence of fluorescent foci. -, absence of fluorescent foci. ND, Not Determined.

Background	$\Delta \text{porV } halo::sprB$	$halo::sprC$	$\Delta \text{porV } snap::sprC$ $halo::sprB$
WT	+	+	+
$\Delta gldJ$	ND	+	ND
$\Delta gldKLMNO$	ND	+	ND
$\Delta sprD$	+	+	ND
$\Delta sprCD$	+	ND	ND
$gldJ_{548}$	-	+	ND

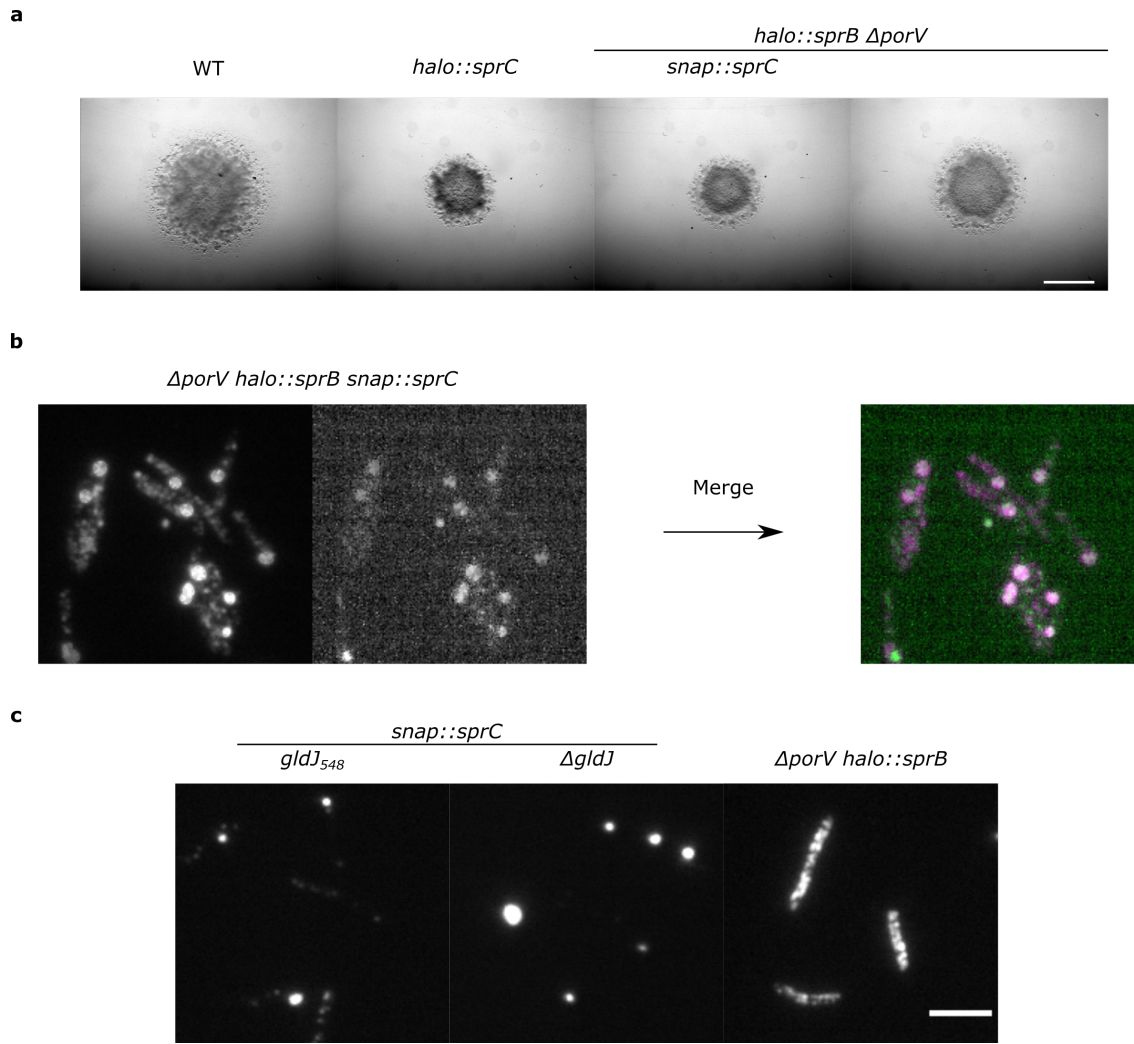


Figure 5.2: Overexpression of the C-terminal 1154-1588 SprD residues result in accumulation of SprC and SprB. **a** Colony spreading of the indicated mutants assessed on PY2 plates. The scale bar represents 5 mm. **b-c** All strains carried a plasmid that overproduced the C-terminal fragment of SprD (SprD₁₁₅₄₋₁₅₈₈), controlled by an *ompA* promoter native to the *F. johnsoniae* chromosome. The indicated strains were grown as described in 2.4.1 and imaged as described in 2.4.2. *ΔporV halo::sprB snap::sprC* were labelled with 100 nM JF646-Halo and 1 μ M JF525-SNAP whereas *halo::sprC* in *gldJ₅₄₈* or *ΔgldJ* backgrounds, and *ΔporV halo::sprB*, were labelled with 100 nM JF646-Halo. Scalebar indicates 5 μ m.

5.2.3 The *sprB* operon is only present within *Flavobacteriaceae*

The *sprB* operon proteins play an essential role in *Bacteroidetes* gliding, by controlling attachment/detachment from the substratum. Since this function is fundamental to gliding my expectation was that these proteins should be universally conserved amongst gliding *Bacteroidetes*. To test this hypothesis, I decided to identify *sprB* operons in refseq genomes of the *Bacteroidetes* phylum (5.3).

To identify *sprB* operons I needed a method of identifying the *sprB* operon genes in each genome. This can be done by representing genes with Hidden Markov models (HMMs). These HMMs can then be used to search genomes for genes that match the HMM. HMMs were retrieved from TIGRFAM for *sprF* (TIGR03519) and *sprB* (Type B CTD, TIGR04131) (Li et al., 2021). HMMs for *sprC*, *remG*, and *sprD* were unavailable and hence build manually. To do this, the Uniprot KB database was searched with HMMER using the *F. johnsoniae* protein sequence as a query sequence. The resulting hits were then used to build a HMM as described in 2.7.1.

Because *sprF*-like genes are often found next to Type B CTD-encoding genes, are required for SprB export, and is thought to link the adhesin to GldJ, I decided to use the *sprF* HMM to identify an initial list of genomes. These genomes were further subsetted to only include genomes in which *sprC*, *sprB*, and *remG* homologs all were within a 5-gene distance of *sprF*. These subsetted genomes was classified as *sprB* operon containing genomes. The *sprB* operon containing genomes were further searched for the remaining *sprB* operon

genes (*sprD* and *remF*) as well as Type A-CTD encoding genes in the proximity of the initially identified *sprF* gene (Figure 5.3). The *sprD* HMM were too inaccurate to identify *sprB* operon regions on its own, and so this technique allowed me to identify *sprD* genes only in proximity to the previously mentioned *sprB* operon genes.

Querying the NCBI taxonomy database with the list of *sprB* operon encoding genomes, a minimal phylogenetic tree that connects the identified genomes was made. The tree was annotated with a 50 kilobase stretch centred around the identified *sprF* gene and the additional *sprB* operon components were colour coded.

The *sprB* operon is only present in a subset of *Flavobacteriaceae*. A separate group encoded Type A CTD-encoding genes next to SprF which also matched the SprB repeat domain and the SprC HMM. These genomes also had a *remG* in proximity to *sprF*. They did not encode a second *sprF*-like gene suggesting that it is not a true *sprB* operon.

The organisation of the remaining genomes show that the *sprB* operon organisation is similar. However, *remF* is not found in all of them. This is not surprising, since in *F. johnsoniae* a paralog (*remH*) is present in the genome. In other species, such redundant proteins might result in the eventual selection of *sprB* operons without the RemF protein encoded in close proximity and therefore would be excluded from this analysis.

sprD was identified in most genomes in addition to β -barrel-only *sprF*-like gene. This suggests that the SprB operon indeed requires both a regular *sprF* gene as well as an adapted *sprF* gene that encodes a further periplasmic region (*sprD*). This finding agrees with the observation that the SprD C-terminal region is required for SprB-mediated adhesion.

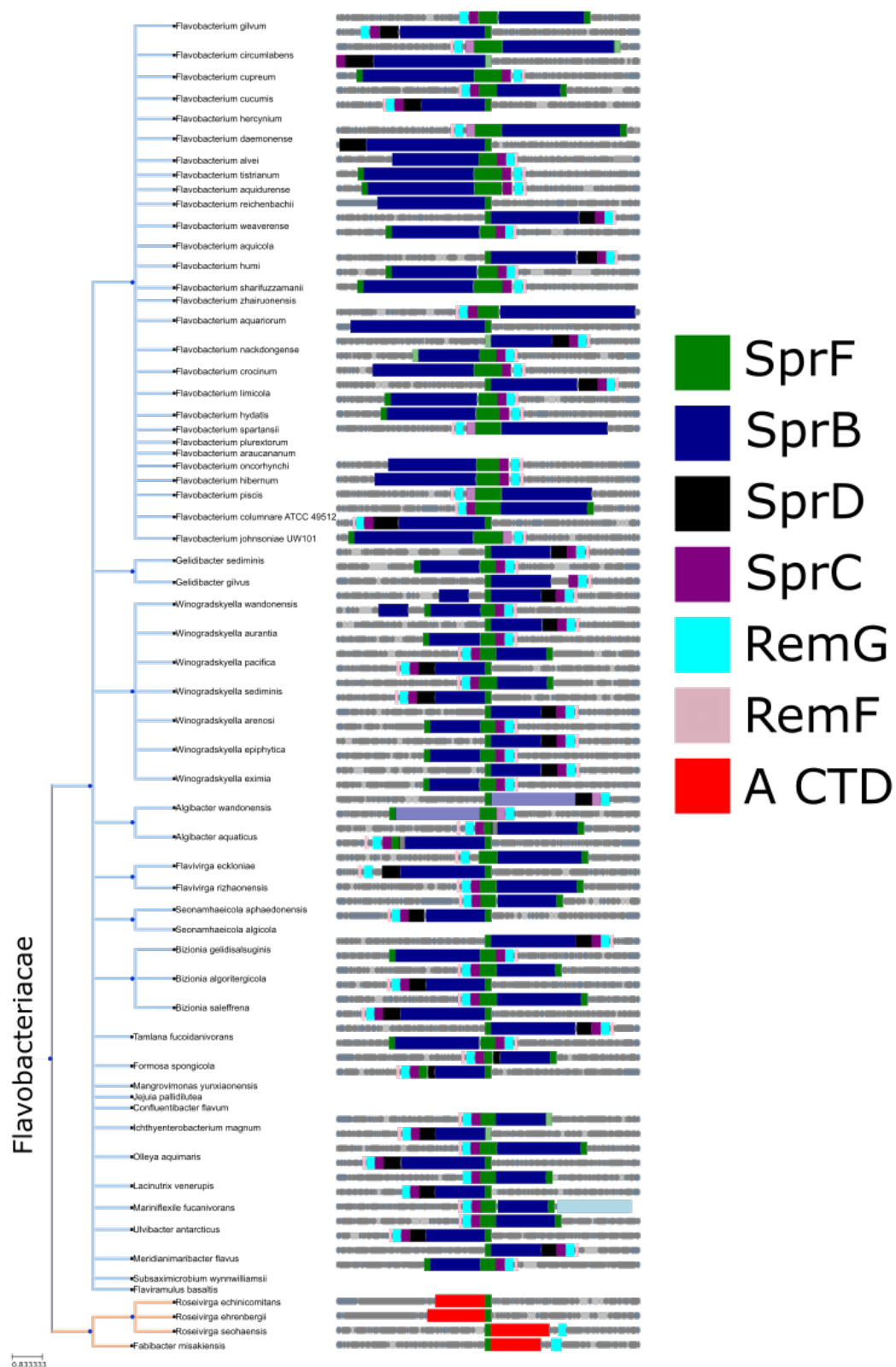


Figure 5.3: Phylogenetic tree of the *sprB* operons in *Bacteroidetes*, annotated with the genomic operon organisation. RefSeq genomes were downloaded from NCBI and the genomes with annotated coding sequences were selected. The *sprB* operon genes were identified by searching the genomes with hidden Markov models (HMMs). SprB and SprF HMM models were downloaded from TIGRFAM whereas HMM profiles for SprC, RemG, and RemF, were created, as described in 2.7.1. Genomes were searched for *sprF* genes. From these genomes, the 5 genes up- and downstream of *sprF* were searched for homologs of all of *sprC*, *sprB*, and *remG*. Genomes with *sprB* operons represented by the presence of all of *sprF*, *sprC*, *sprB*, and *remG* were selected. The taxonomy ID of these genomes were used to query the NCBI taxonomy database to extract a minimal tree representing the selected genomes. The tree was annotated with a 50kb stretch from the selected genomes, and the genes were coloured based on the matching HMM profile. Further colouring of genes matching *sprD*, *remF*, and Type A CTD-encoding gene HMMs were done on the extracted tree. Branch length of the tree has been fixed and does not represent evolutionary distance.

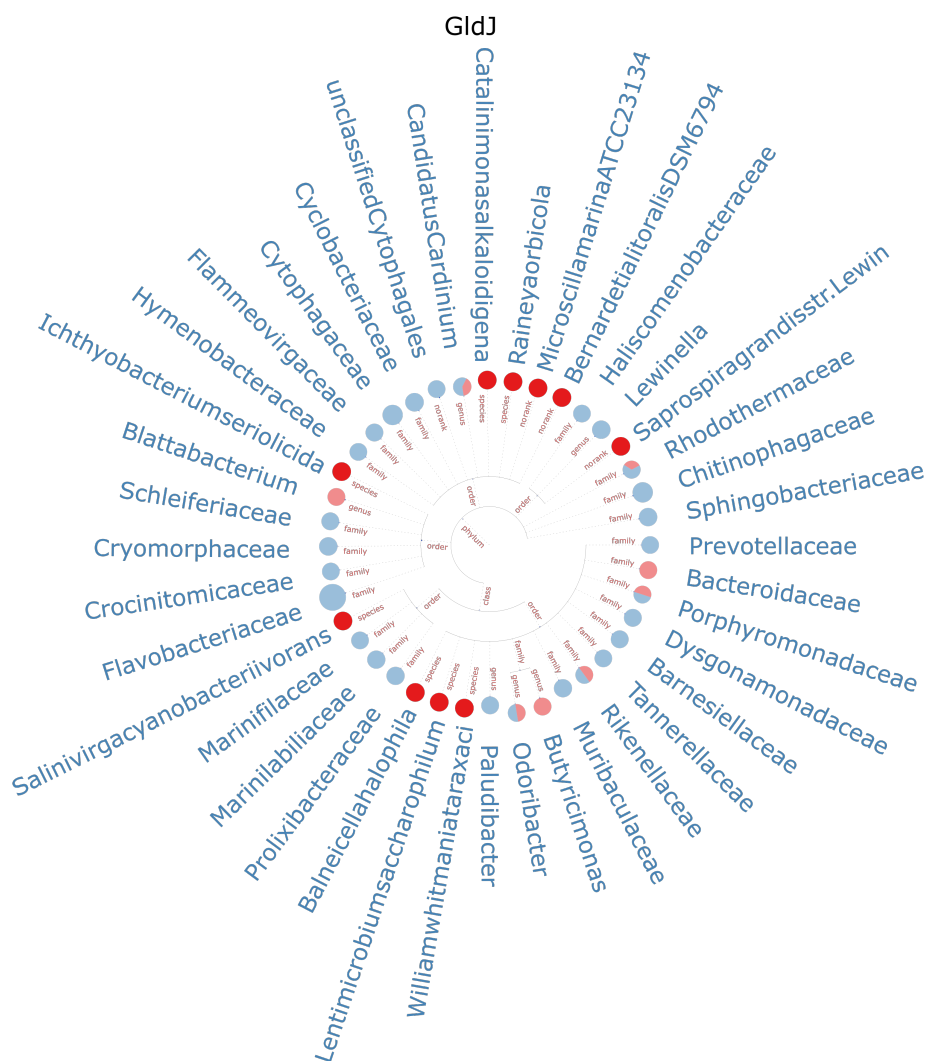
Because the *sprB* operon is not conserved in all gliding *Bacteroidetes*, I decided to study whether a subset of the *sprB* operon genes or domains could constitute a minimal set of genes required to control adhesion of helically moving adhesins in general.

SprF is the most likely protein required for the helical movement of SprB along the GldJ track. In my gliding model SprC, SprD, RemG, and potentially other proteins are thought to move along the GldJ track in a complex that can interact with an SprB-SprF complex to activate adhesion. The movement of the proteins along the track likely depends on the N-terminal domain of SprD (the SprF-like domain). Thus, a minimal *sprB* operon should constitute two helically moving entities that both depend on SprF-like proteins to work. To test if such a minimal *sprB* operon exists in the broader distribution of gliding species, I started by identifying gliding species in *Bacteroidetes*. Species whose genomes encode *gldJ* are assumed to be capable of gliding motility. When searching only for *gldJ*, a

broad conservation across most of *Bacteroidetes* (Figure 5.4, *a*). Using the same tree to draw the distribution of *sprB* operon containing genomes (Figure 5.3), it was confirmed that this operon is not present in all gliding species, but rather only in *Flavobacteriaceae* (Figure 5.4 *b*). I then used the same tree to analyse the presence of two *sprF* genes in genetic proximity to one another. Using the *sprF* HMM, I retrieved genomes with a *sprF* gene, and searched the 5-gene neighbourhood for a second *sprF* gene, similar to the approach described above for the *sprB* operon identification. The distribution still seemed mostly confined to *Flavobacteriaceae*, and although other species outside this branch had two *sprF*-like genes in close proximity, the distribution was far less extensive than *gldJ* (Figure 5.4 *c*). It is possible that *sprF*-like genes do not lie in such proximity and thus would not have been identified. However, because *sprF*-like genes often lie in proximity with their respective Type B CTD encoding target gene, I hypothesised that these genes would not be able to compensate each other in most cases (Kulkarni et al., 2019). *sprF*-like genes hence does not seem to be sufficient to constitute a minimal system, if such a minimal system exists.

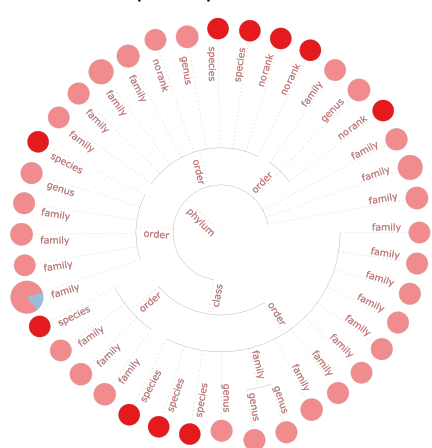
In summary, the *sprB* operon is not conserved across all gliding *Bacteroidetes* species. The *sprB* operon is indispensable for gliding under most conditions studied in the laboratory. Thus, there must be other proteins that perform a similar function in gliding species without *sprB* operons.

a



b

SprB SprC RemG



c

SprF x2

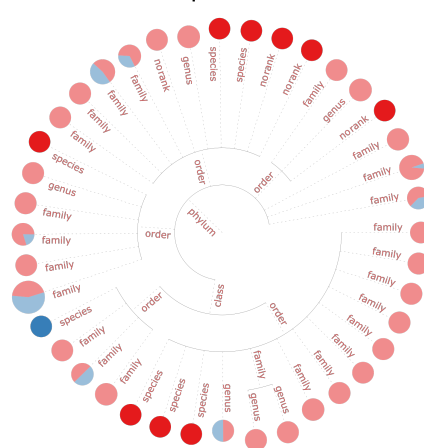


Figure 5.4: Distribution of *gldJ* and *sprB* operons in *Bacteroidetes*. The taxonomy IDs of all annotated *Bacteroidetes* RefSeq genomes were used to query the NCBI taxonomy database and used to produce a minimal tree. The branches were collapsed for visibility and represented by a pie chart. The size of each pie is scaled according to the number of strains collapsed under the node. Pies with a darker colour represents a single species and thus no collapsed species are under these nodes. The phylogenetic tree in **a** is identical to **b-c** and so the names are only drawn on **a** to conserve space. (a) Distribution of *gldJ* in *Bacteroidetes*. *gldJ* was identified in the genomes using the TIGR03524 HMM. Species with *gldJ* were coloured blue and those without were coloured red. Under collapsed nodes, the pie diagram represents the proportion of subspecies under the node that encoded *gldJ*. (b) Distribution of *sprB* operon, identified by the presence of *sprB*-, *sprC*-, and *remG*-like genes near *sprF* as described in Figure 5.3. (c) Distribution of a hypothesised minimal *sprB* operon, consisting of two *sprF*-like genes in close proximity. Similar to **a**, *sprF*-like genes were also identified using HMM matching (TIGR03519).

5.2.4 GldJ interacts with the T9SS and proteins encoded in the major adhesin operon

If GldJ is indeed forming the gliding motility track, it would need to interact with components of the motor as well as the adhesins. I decided to perform *in vivo* cross-linking mass spectrometry (CXMS) to identify the proteins that interact with GldJ. A mutant strain producing a C-terminal His₆ tagged GldJ protein was made by Frederic Lauber and shown to be gliding similar (spread ~90% diameter of wt on PY2 agar plates) to wt (data not shown, and Frederic Lauber personal communication). Cells expressing GldJ-His₆ were grown under motility promoting conditions and cross-linked *in vivo* with the homo-bifunctional N-hydroxysuccinimide-ester di-succinimidyl-suberate (DSS) or treated with a control solution of Dimethyl sulfoxide (DMSO). DSS crosslinks primary amines

such as the ones found on lysine residues. GldJ-partner complexes were then isolated by Ni-NTA purification from cell extracts solubilized in n-Dodecyl- β -D-Maltoside (DDM) and urea. Immunoblotting analysis of the elution fractions revealed several bands with higher molecular weight than monomeric GldJ-His₆ in the cross-linked sample indicating that GldJ-containing adducts had been formed (Figure 5.5 A). Control and cross-linked elution samples were digested with trypsin and analysed by electrospray ionisation mass spectrometry (ESI-MS).

Figure 5.5: Crosslinking mass spectrometry of GldJ-His₆. Cells expressing GldJ-His₆ were grown as described in 2.3.5 and cross-linked with DSS *in vivo*. GldJ-partner complexes were then isolated by Ni-NTA purification from cell extracts solubilized in DDM and urea. (a) DMSO control and DSS cross-linked samples were eluted 4 times from the Ni-NTA beads and subjected to Western blot analysis using α His antibody. (b-c) Volcano plot (c) of detected proteins colored by subcellular localisation according to the colour legend in b. The second DMSO control and DSS elution fraction were digested with trypsin and analysed with ESI-MS. The experiment was performed twice, with two replicates the second time. Proteins not identified in either control or crosslinked datasets across all repeats were dropped. The remaining proteins which were not present (i.e. in cases where a protein was only present in e.g. control but not crosslinked) had their intensity set to the minimum value of their respective datasets. The intensity of the datasets were normalised in R using limma (Smyth, Speed, 2003), transformed to log₂ scale, and the *p*-value for each protein calculated with Student's *t* test. Log₂ fold change between the cross-linked and DMSO treated samples were plotted against the *p*-value. The proteins, represented as points, were coloured by their subcellular localisation using psortb predictions (Yu et al., 2010) and, where possible, manual curation from the literature. Red boxes indicate mutants further analysed in Figure 5.6. Horizontal line indicates a *p*-value of 0.05. Names of proteins of interest were shown.

In support of the hypothesis that GldJ interacts with both the T9SS and helically moving proteins, the experiment identified OM-localised T9SS core proteins GldK (log₂ 5.20, *p* 0.002), GldN (log₂ 4.31, *p* 0.001), and GldO (log₂ 6.29, *p* 0.008) as well as *sprB* operon proteins. SprF was less enriched (log₂ difference 1.86, *p* 0.08) than SprB (log₂ difference 5.94, *p* 0.009), although it is thought that SprF bridges the OM to connect SprB with GldJ. One possibility is, that the crosslinked peptides had poor ionisation properties and were not readily identifiable in mass spectrometry. Alternatively, the sensitivity was too low in general to detect SprF. Finally, the barrel of SprF could have been shielded by the OM, reducing the chance of forming crosslinks between GldJ and SprF. This is less likely

since several other β -barrels were identified. SprD ($\log_2$ 4.79, p 0.054) and SprC ($\log_2$ 5.48, p 0.057) were also identified, adding another two helically moving proteins to the interaction partners of GldJ.

Several other envelope proteins were enriched by crosslinking to GldJ that did not have an assigned function (Figure 5.6, **a**). I was successful in deleting some of the genes encoding these proteins (red boxes, Figure 5.5) and I assessed the colony spreading phenotype on PY2 agar (Figure 5.6, **a**). Only one of these genes was required for wild type colony spreading: *fjoh_5040*. This gene encodes a small (194 residues) protein of unknown function with no predicted domains apart from a signal peptide. Since Fjoh_5040 influenced colony spreading, I decided to investigate the in-cell mobility of this protein by fusing a HaloTag domain to the protein and imaging it using HILO microscopy. The fusion protein did not move helically but rather randomly with a speed indicative of a periplasmic protein (Figure 5.6, **c**). However, the strain expressing the fusion protein phenocopied the Δ *fjoh_5040* mutant in being unable to support colony spreading on PY2 plates (data not shown) suggesting that the HaloTag fusion protein was non-functional. Consequently, I did not study this fusion further. A reduction in spreading might result from attenuated helical movement of SprC or SprB. To test this, I deleted *fjoh_5040* in the *halo::sprC* and Δ *porV* *halo::sprB* backgrounds but characteristic helical motions were still observed for both proteins (Figure 5.6 **c**).

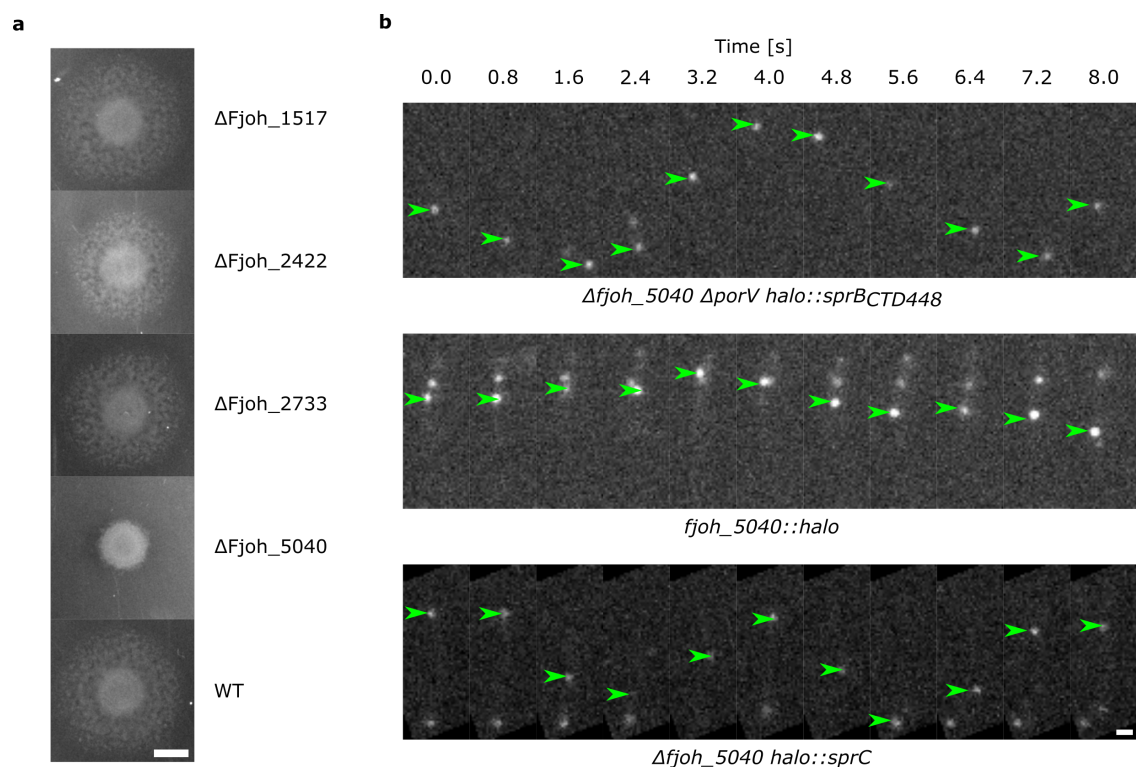


Figure 5.6: Role of CXMS candidate genes in gliding motility. **(a)** Colony spreading on PY2 agar plates of selected mutants from Figure 5.5 **c**. Scalebar, 2 cm. **(b)** Assessment of helical protein movement. The indicated strains were grown as described in 2.4.1 and imaged as described in 2.4.2. *fjoh_5040::halo* cells were labelled with 100 nM JF646-Halo and imaged without strobing. $\Delta porV halo::sprB_{CTD448}$ and *halo::sprC* were labelled with 10 nM PAJF646-Halo and strobed with a 1 on 3 off cycle. Every 10th ($\Delta porV halo::sprB_{CTD448}$ and *halo::sprC*) or 40th (*fjoh_5040::halo*) frame are shown from each movie corresponding to a timestep of 800 ms. Green arrowheads indicate the position of the helically ($\Delta porV halo::sprB$ and *halo::sprC*) or Brownian diffusing (*fjoh_5040::halo*) molecule. Scalebar, 1 μm .

5.2.5 The gliding motility proteins GldD and GldB move in a helix

Because the *sprB* operon proteins are not produced by many gliding species, these proteins may be a specialised adaption in the *Flavobacteriaceae* to optimise the gliding machinery for their specific environmental niche. This suggests that other more widely conserved gliding proteins have more fundamental roles in the gliding mechanism. To test this, I decided to investigate the gliding-specific OM lipoproteins which comprise GldDBHL. These proteins are essential for gliding. RNA-seq data suggest that GldJ is more abundant than all the other gliding specific lipoproteins, in accordance with the hypothesis that it forms the track (Baez et al., 2019). Therefore, the other gliding proteins could be involved in modulating the functions of the track, which might require them to move along the track.

To test this idea, I decided to fuse HaloTag to the gliding lipoproteins and track them using the 2D SPT methodology described in chapter 4. I was successful in creating chromosomal fusions of the *halotag* sequence to *gldD* and *gldB*. These mutants were capable of spreading on PY2 agar plates (Figure 5.7, **a**). Immunoblotting of the mutants shows that the fusion proteins were stable and uncleaved (Figure 5.7, **b**). HILO microscopy revealed helical motion for both Halo-GldB and Halo-GldB producing mutants (Figure 5.7).

However, even though the fusion proteins are functional, most of the fusion proteins were immobile or moved very little, complicating straightforward interpretations of the roles of these proteins in gliding motility.

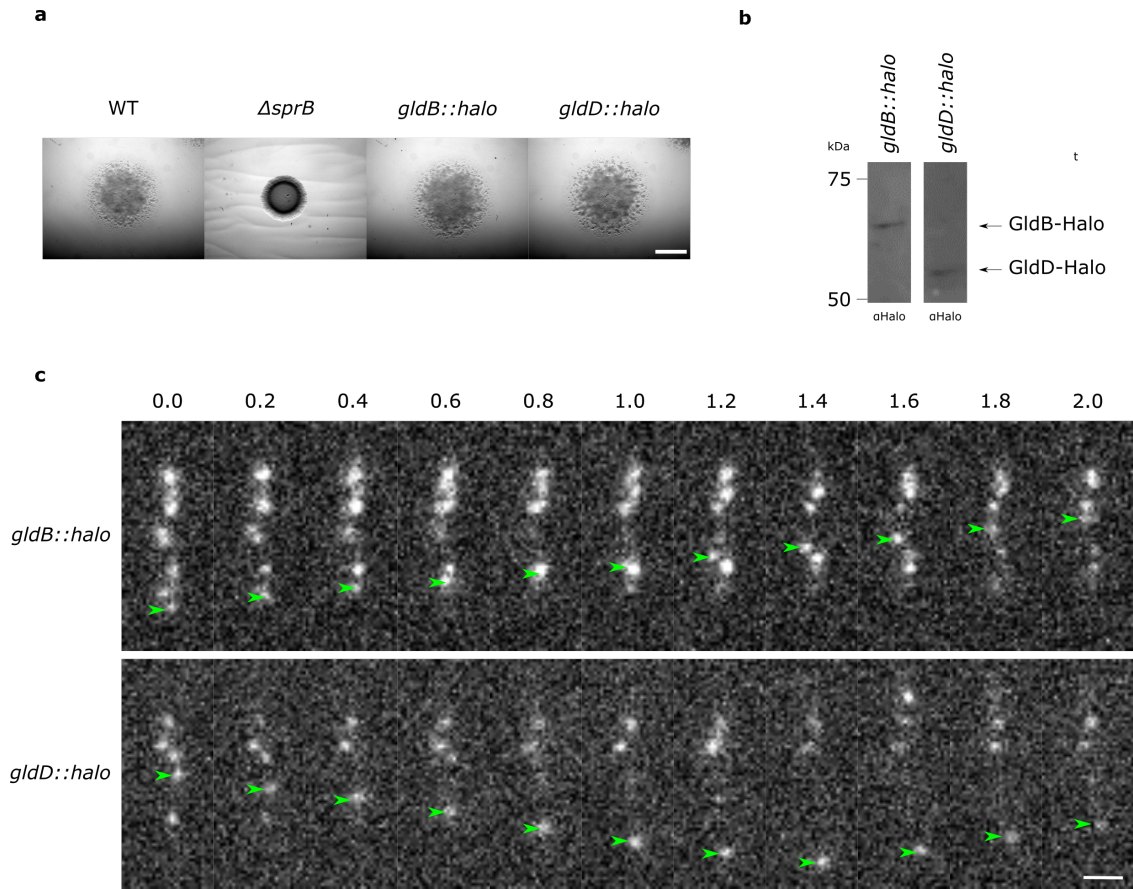


Figure 5.7: GldD-Halo and GldB-Halo can move in a helix. **a** Colonies of the indicated strains grown on PY2 agar plates for 24 hr. Scalebar, 5 mm. **(b)** Immunoblot of the indicated strains, grown as described in 2.3.2. **(c)** Representative images from microscopy movies of helically moving molecules. The indicated strains were grown as described in 2.4.1, labelled with 100 nM JF646-Halo and imaged as described in 2.4.2 without strobing. Each image represents every 10th frame of the movie. Green arrowheads indicate a helically moving molecule. Scale bar, 2 μ m.

5.3 Discussion

The gliding machine of *F. johnsoniae* is divided between the daughter cells upon cell division. It was more than 2 decades ago, that cells were observed to temporarily halt gliding

for 1-5 min immediately before cell-separation (Kempf, McBride, 2000). The authors reported that the cells resumed gliding immediately after cell division, thus suggesting that the components required for gliding were located in both daughter cells. This poses a challenge for proteins such as SprC, which we know from Chapter 4 localises to the leading pole during active gliding. However, the fact that cells would halt suggests that SprC would not be directing active gliding during this period. Indeed, when observing Halo-SprC in dividing cells, I frequently observed mid-cell foci. This suggests that SprC, like SprB, is localised to the mid-cell leading up to cell division and so the cells could no longer glide actively.

In this chapter, I show that SprB requires GldJ for midcell focus formation. In accordance with this observation, GldJ has previously been shown to accumulate near division sites (Braun, McBride, 2005). Thus I hypothesise that GldJ informs the helically moving proteins about the cellular status. CXMS experiments support this notion, as several helically moving proteins were found as interaction partners of GldJ.

Overexpression of an SprD C-terminal fragment, like cell division, also caused SprB to accumulate in foci, in a GldJ-dependent manner, though in this case anywhere on the cell body. The observation that GldJ was required for this process could mean that the *sprB* operon proteins are acting through GldJ to influence adhesion.

Finally, the CXMS data indicates that GldJ also interacts with the core T9SS proteins GldKNO which are hypothesised to form a large ring structure at the periplasmic leaflet of the outer membrane (Gorasia et al., 2020)(Gorasia et al., 2016). Thus GldJ interacts with both the T9SS and helically moving *sprB* operon proteins. This interaction network

is in accordance with the rack and pinion hypothesis, wherein the rack (GldJ) moves along a helical track, driven by pinions (T9SS). The potential interface for energy transfer between the T9SS and the track could thus be GldJ-GldK (Shrivastava, Berg, 2020).

The *sprB* operon is required for *F. johnsoniae* gliding under our laboratory conditions. By searching for the *sprB* operon structure in all *Bacteroidetes* genomes available via RefSeq, I found that the *sprB* operon is not conserved outside *Flavobacteriaceae*. Reducing the stringency of my search by representing the *sprB* operon as a minimal version with two *sprF*-like genes in the proximity of one another did not broaden the conservation of genomes enough to suggest that the *sprB* operon model is a universal mechanism for the control of gliding. Thus, it seems that the *sprB* operon is a highly evolved and specific invention of the *Flavobacteriaceae*. Future studies are needed to understand what advantage has driven this evolution and how directional movement is controlled in other gliding *Bacteroidetes*.

Because the *sprB* operon is not universally required for gliding motility, I turned my attention towards the genes conserved and co-occurring with *gldJ*. Since gliding cells still require a way of selective attachment, I rationalised that such control proteins should be moving in a helix, similar to the *sprB* operon encoded proteins, or as a minimum be localised to the periplasmic leaflet of the OM. I found that GldD and GldB both are capable of helical movement. Most of the GldD and GldB proteins were immobile or moving very little during imaging, unlike SprB. The copy number is also estimated to be much lower. GldB and GldD both lack obvious homologs, and it was not possible to get a structure prediction with high confidence. Both proteins are known to be lipoproteins, and

so they are the first gliding lipoproteins shown to move in a helix. Curiously, only GldB was identified in the CXMS dataset and did not show high enrichment in the crosslinked fraction ($\log_2 2.02$, $p 0.047$). I do not have enough data to assign roles to GldD and GldB. Speculatively, the lower copy number relative to GldJ and the ability to move in a helix suggest that these proteins could be important in directing GldJ topology or otherwise regulating track formation.

Chapter 6

Conclusion and future perspectives

6.1 3-dimensional single-particle tracking of SprB

Gliding motility in the *Bacteroidetes* involves the movement of cell surface adhesins on an internal track, which current models propose to be formed by the lipoprotein GldJ. GldJ is essential for gliding motility and universally conserved in motile, but not non-motile, *Bacteroidetes* species (McBride, Zhu, 2013). Other highly conserved gliding-specific genes that are almost always present in *gldJ*-encoding genomes are required for the post-translational stability of GldJ (Johnston et al., 2018). Thus, GldJ seems to be the central protein with which most of the other gliding components interact. GldJ is homologous to the T9SS core protein GldK, a lipoprotein anchored to the periplasmic leaflet of the OM where it forms large (50 nm diameter) rings with the periplasmic protein GldN, as suggested from *P. gingivalis* experiments (Gorasia et al., 2016). RNA seq data suggest that the *gldKN*

operon and *gldJ* is about 10-fold more highly expressed than the other gliding specific genes (Baez et al., 2019) consistent with the hypothesis that GldJ, and possibly GldKN, form the OM track that powers and guides the movement of adhesins such as SprB. Using immunoelectron and immunofluorescence microscopy, Braun, McBride (2005) reported that GldJ had a helical organisation in fixed cells as expected of the track upon which helically moving adhesins travel. However, the localisation and dynamics of GldJ have not been visualised in alive and moving cells. The initial aim of this project was to develop a methodology that would allow me to visualise the GldJ track in living cells to explore the distribution and dynamic nature of GldJ. Unfortunately, despite extensive efforts, I was unable to introduce a fluorescent tag that would allow me to observe GldJ in live cells. All attempts yielded proteolysed GldJ, therefore I Instead decided to fluorescently tag SprB and let the tracking of fluorescent SprB molecules 'draw' the outline of the underlying track through single-particle tracking (SPT).

In chapter 3, I developed a 3D SPT methodology that allowed tracking of SprB on the minute timescale. Previous work had inferred that the helical trajectory of SprB formed a closed-loop (Nakane et al., 2013)(Shrivastava, Berg, 2020). Using my methodology I was able to follow SprB molecules through several runs along the cell length. This showed that the track is not a single closed helical loop. Instead, it seems that SprB can move in a helical path almost unrestricted by the location on the cell body. Thus, either many tracks are present at the same time, or there are dynamic tracks that can move along most areas of the cell surface. The dynamic tracks are hypothesised to follow approximately helical paths either guided by a helical object such as the cell wall or the curvature of the

membrane itself.

In chapter 3, I suggest a treadmilling model, wherein GldJ forms dynamic filaments that connect to T9SS complexes distributed at discrete locations throughout the cell body. This model helps to explain several observations that were incompatible with the closed-loop model.

First, I observed SprB molecules that moved at different speeds within the same cell, for the same period of time. Although they could slow down or speed up as a result of how well they are connected to a fixed and energised track, the smooth and gradual increase and decrease in SprB speeds make this scenario less likely as a more pronounced step-wise change would have been expected in that case. In the treadmilling model, the SprB molecules would depend on the treadmilling speed of individual track patches which are energised by the locally connected T9SS and therefore allow individual speeds of several SprB molecules within the same time.

Second, I observed SprB trajectories that cover most of the cell surface, illustrating that there is more than a single track present. This was further supported by the observation that multiple SprB molecules could move along different paths from the leading to the lagging pole, at the same time. If there was a single closed loop, all molecules had to move along the same path. Due to technical limitations, only a limited number of SprB molecules can be observed in a single cell at the same time. Thus, I was unable to confidently show that more than 4 tracks were leading from one pole to the other, at the same time. However, given that there are at least 4 active tracks at the same time, it seems more likely that fewer tracks would have the ability to move or disassemble and

reform in new regions rather than many fixed tracks coexisting. Unfortunately, my data did not allow me to visualise the potential track movement itself, and so resolving this issue will need to wait until a method of observing GldJ movement is developed (discussed further below, in 6.6).

6.2 The role of the *sprB* operon in gliding motility

Gliding motility in *Bacteroidetes* depends on helically moving adhesins. Because the adhesins are moving both towards and away from the leading pole of the cell, they must be able to let go of the substratum when they are moving towards the leading pole and attach only when moving away from the leading pole. If adhesins are unable to let go of the substratum, a cell would continuously move over the area of adhesion, never moving further than a cell length. This phenotype has been demonstrated for multiple *sprB* operon deletion mutants (Rhodes et al., 2011a). The second aim of this project was to study if and how the adhesion of the SprB was controlled. SprB is absolutely required for active gliding under our laboratory conditions. However, there is no experimental evidence that the adhesiveness of SprB can be regulated even though this is a fundamental requirement of the current gliding model.

In chapter 4, I show that none of *remF* and its homolog *remH*, *remG* and its homolog *remI*, *sprC*, or *sprD* were required for helical movement of SprB. Rather, null mutations in these genes affected the SprB-mediated adhesion to glass. Thus, I hypothesised that the *sprB* operon components are involved in the regulation of SprB adhesion to the substratum.

Because of the speed of SprB movement, I hypothesised that this regulation had to occur over a short time period. One method of quickly regulating SprB adhesion could be through direct interaction of the *sprB* operon proteins with SprB. SprF is thought to interact directly with SprB as it is required for the secretion of SprB through the T9SS. Given the structural similarities between SprF and PorV, SprF likely facilitates secretion similar to what is thought for PorV. PorV binds to the SprA barrel protein and interacts with the substrate as it is secreted through this barrel. Since SprB is surface-localised, it is hypothesised that the SprF-SprB binding, established through secretion, remains after secretion. This hypothesis is in line with the evidence that the presence of SprB protects SprF from proteinase K proteolysis (Kulkarni et al., 2019).

Using the 3D SPT assay developed in Chapter 3, I showed that SprC, RemG, and SprD all move helically, similar to SprB. Interestingly, these proteins are periplasmic and outer membrane proteins rather than cell surface proteins like SprB. SprD encodes an SprF-like N-terminal β -barrel membrane domain which is sufficient to enable helical movement of SprC and SprD. The C-terminus of SprC is distantly related to the CTD of T9SS substrate proteins that are used to target the protein for export. Thus, it seems that SprC and SprD constitute a distant SprF-like protein and CTD containing protein pair similar to SprF-SprB. Curiously, SprC and SprB are on opposite sides of the OM so it is unlikely that they interact with their SprF-like partner protein in a similar way. There is no experimental evidence to support that SprF can move in a helix and HaloTag fusions of SprF and PorV have rendered them non-functional (Xiolong Liu, personal communication). However, the observation that a similar domain is present in a fragment of SprD (SprD₁₋₆₃₃) that

can move helically and that a shorter fragment containing the SprF-homologous region (SprD₁₋₃₀₉) is sufficient to support SprC helical movement supports the hypothesis that SprF itself is capable of helical movement. Curiously, immunoblotting shows that Halo-SprC runs at a smaller size in the absence of the SprF-like N-terminal domain of SprD, suggesting that this domain is involved in the post-translational processing of SprC or that it protects SprC from proteolysis. Future research is needed to determine the significance of this observation.

In addition to imaging the Halo-SprC fusion in cells immobilised on agar pads, I also imaged cells gliding on glass slides. Under these conditions, I found that SprC accumulates at the leading pole of actively gliding cells. This accumulation only occurred when the cells were capable of motion, as depletion of the oxygen used as a respiratory electron acceptor or addition of a protonophore caused the cells to halt SprC movement and resulted in the protein being randomly and equally distributed throughout the cell. This happened gradually as the cells lost energy, suggesting the accumulation of SprC is dependent on the internal energy state of the cell. SprB does not accumulate at the cell pole of gliding cells and so it seems SprC is able to move differently than SprB, despite both moving on helical tracks. The C-terminal end of SprD is required for gliding and a 50 residue C-terminal truncation of the 1588 aa protein phenocopies $\Delta sprD$ (Rhodes et al., 2011a). In this thesis, I show that these C-terminal 50 residues are not necessary for the helical movement of SprC and SprD, but instead are required for the polar accumulation of SprC in gliding cells and SprB-mediated adhesion to glass. Since the SprB-mediated adhesion to glass could be a pre-requisite for polar accumulation of SprB and *vice versa* I was

unable to assign a specific role for these residues.

6.3 GldJ as a gliding motility track

The last aim of this project was to verify experimentally that GldJ interacts with both the T9SS and adhesins as predicted by my and other models of gliding motility. Unfortunately, I was unable to purify GldJ and found that I was only successful in solubilising the protein when using 8M urea. To detect GldJ-interacting proteins I, instead, opted for crosslinking mass spectrometry (CXMS), as this would identify proteins in close contact with GldJ and thereby reveal partner proteins such as the T9SS and adhesins.

In chapter 5, I present CXMS data that show GldJ interacts with GldK, GldN and SprB. Unfortunately, the experiment was not successfully reproduced a 3rd time due to lack of time and challenges in the experimental procedure. Nonetheless, the data support a model in which GldJ interacts with the T9SS through the OM lipoprotein GldK. It is not clear why SprF, which is hypothesised to link external SprB to the internal GldJ track, was not identified when SprB was, but this likely reflects a methodological issue rather than a deficiency in our molecular model for gliding.

6.4 The updated gliding motility model

Taken together, the data presented in this thesis can be summarised into an updated working model for *F. johnsoniae* SprB-mediated gliding motility (6.1). The *sprB* operon encodes proteins that can regulate the ability of SprB molecules to adhere to the sub-

stratum. SprC, and potentially other *sprB* operon proteins, localise to the leading pole in actively gliding cells. This polar localisation, possibly mediated by the C-terminal end of SprD, informs the motility machinery of the direction of movement. Since the N-terminal SprF-like domain of SprD is required for SprC helical movement, I theorise that SprC and SprD interact with GldJ in a complex. This complex encounters helically moving SprB-SprF and a transient SprCD-SprBF complex is formed with activated SprB adhesion. This SprCDBF complex moves in a helix from the leading to the lagging pole, where some detachment mechanism occurs that stops SprB adhesion. In my model, I suggest this be mediated by dissociation of the SprCD and SprBF complexes from one another, but it could just as well be through other unidentified mechanisms such as polar accumulated proteins at the lagging pole.

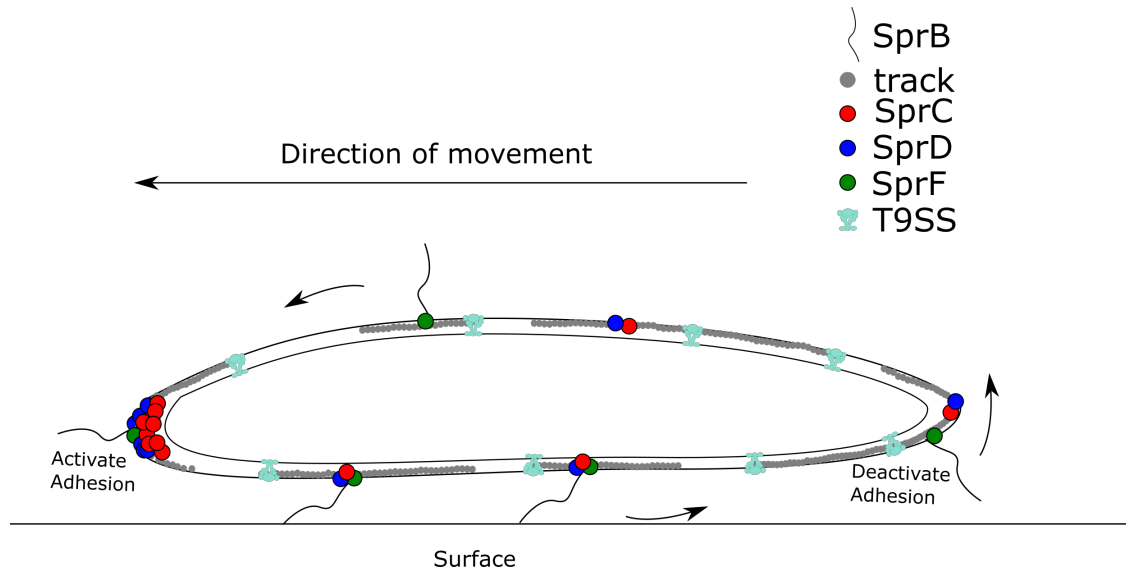


Figure 6.1: Updated model of *F. johnsoniae* SprB-mediated gliding motility. The *sprB* operon proteins SprC, SprD, and likely others, localise to the leading cell pole. Helically moving SprF-SprB complexes pass by the leading pole and form a transient complex with SprD and SprC. The SprCDFB complex has SprB-adhesion activated while it moves along, or with, treadmilling GldJ. The treadmilling is powered by the T9SS, located at distinct spots around the cell body. Through an unknown mechanism, SprCD dissociated from the SprBF complex near the lagging pole while both continuing to move on or with the helical track. This dissociation results in loss of SprB adhesion and both subcomplexes move back to the leading pole where the process can restart.

Expanding the *F. johnsoniae* SprB-mediated gliding motility model to a more general *Bacteroidetes* gliding motility model, a mechanism of movement that does not involve the *sprB* operon is required. It is likely that a general mechanism is present for gliding motility across all gliding *Bacteroidetes*, and that adaptations such as the specialised *sprB* operon are an improvement upon the standard gliding machinery. Assuming such a scenario, the *Bacteroidetes* gliding motility model should be simple and include only the most conserved elements across all gliding species. To this end, I propose that GldDBHIJ

(track), T9SS (power source), and SprF-like protein or PorV-like protein with one or more associated adhesin T9SS substrate proteins constitute such a minimal system. In this model, the track is constituted of GldJ and the T9SS, assuming the treadmilling model I proposed above, with additional accessory proteins that can move helically and structure or otherwise facilitate the track creation without being an inherent part of it. For example, if GldJ is treadmilling, GldDBHI could form a tip or tail of such a polymerising complex. Most genes encoding SprF-like proteins co-localise with genes that encode T9SS CTD proteins, in the genome. Often, these genes encode adhesins, and so I hypothesise the minimal adhesin complex to be a pair of these two proteins. As stated, the gliding cells must be able to let go of the substratum as the helically moving adhesins move from the lagging to the leading pole. Because the minimal model proposed here does not include the elements of the *F. johnsoniae* gliding motility responsible for this function, I propose that the minimal system instead relies on a tug-of-war model wherein the collective set of adhesins attached to the surface attach more strongly than the single adhesin that is about to let go, so it can rip off and not form stable attachments in the periods where it is in contact with the substratum while moving back towards the leading pole.

6.5 The updated gliding motility model in relation to *M. xanthus* gliding motility

The observation of a polar accumulation of SprC is the first example of asymmetry in *Bacteroidetes* gliding motility and provides the first clue in understanding cell polarity

and navigation in these organisms. In comparison, the polarity and control of directional movement in *M. xanthus* is well understood. A GTPase system composed of MglAB and RomRX ensures that MglA cycles between a GTP bound (RomRX induced) and a GDP bound (MglB induced) state. RomRX-MglA-GTP resides at, and indicates, the leading pole. RomRX-MglA-GTP stimulates and incorporates into the focal adhesion complex (FAC) that moves helically along the cell body via the MreB helical track, powered by the AglRQS motor proteins. MglB and MglA depends on RomR for polar localisation (Keilberg et al., 2012). However, RomR is a response regulator with a Glu-rich tail and although it has not been shown, the domain structure is unlikely to be sufficient for polar localisation on its own (Leonardy et al., 2007). Thus, although not identified yet, I hypothesise that there is a polar determinant in *M. xanthus* with which proteins such as RomR interact. Indeed, since RomRX slowly moves from the leading to the lagging pole (through incorporation into FACs), leading pole MglA-GTP likely interacts with something other than RomR to continue to localise to the leading pole.

Interestingly, the MglAB polarity module is also important for establishing pole identity during sporulation. *M. xanthus* does not form spores similar to the well-studied *Bacillus subtilis* and related firmicute species, as it does not undergo cell division as a means of forming the spore. Rather, the whole cell is transformed into the spore during sporulation (Zhang et al., 2021). Two methods of forming spores are known: 1) spore formation in fruiting bodies and 2) spore formation via treatment with chemical stress. Spore formation via the second method is useful for laboratory study and is often achieved by treatment with glycerol. Glycerol induced spores do not have peptidoglycan (Bui et al., 2009).

Importantly, spores lose the memory of the poles and seem to form new poles at random sites where peptidoglycan synthesis begins upon germination (Zhang et al., 2020). This *de novo* pole formation depends on *mglA* and *mglB* as null mutants of these genes result in vegetating spores showing several bulges suggestive of multiple pole formation (Zhang et al., 2020). Fluorescently labelled MglB form clusters where the new poles are formed, and these clusters co-occur with the sites where peptidoglycan has been synthesized. Following MglB lagging pole formation, the leading pole is indicated by MglA, and the MglA-MglB polarity axis is established. MglA and MglB seem to repel the Rod complex, and the motor proteins AglRQS together with MglA and MreB are expected to move around the cytoplasmic membrane and aid in the distribution of rod complexes along the elongating cell (Zhang et al., 2020). The Rod complex (RodA, PBP2, and MreB) is responsible for the synthesis and elongation of the peptidoglycan in rod-shaped cells (Teeffelen van, Renner, 2018). As mentioned earlier, MreB forms the helical track in *M. xanthus* and behaves differently from the canonical MreB in other rod-shaped bacteria such as *B. subtilis*. Therefore, the relationship between the polar MglA and MglB, and the Rod complex is nicely explained by MreB as a common component.

Interestingly, a null *romR* mutant did not have near as pronounced effect on polarity misplacement as the *mglA* and *mglB* mutants, supporting the hypothesis that there is another element responsible for polar anchoring (Zhang et al., 2020). Since the peptidoglycan has been synthesised at MglB-defined poles, the unknown polar determinant may be a central element of cell growth such as peptidoglycan synthesis. In the polarly flagellated *Vibrio* species, the polar protein HubP targets multiple cell processes to the pole such as chemo-

taxis proteins, the chromosome, and flagella (Yamaichi et al., 2012). HubP consists of two domains, a LysM periplasmic domain and a cytoplasmic domain. The periplasmic domain likely interacts with the peptidoglycan and is required for the polar localisation (Yamaichi et al., 2012). Thus, it seems that polar determination by interaction with the peptidoglycan is not an uncommon mechanism for the identification of the pole.

In *F. johnsoniae*, SprC localises to the pole during active gliding. Thus, a polar landmark should aid in this localisation process. HubP and the MglAB and RomRX proteins mentioned above all had motility defects and were not essential, making identification of them by deletion possible. However, the common theme seems to be that they are guided by the peptidoglycan layer. Thus, I propose that for *F. johnsoniae* the cell wall or associated proteins could be important for this polar anchor. Specific inward peptidoglycan synthesis often occurs near the division site at the time of division (Surovtsev, Jacobs-Wagner, 2018). If the polar identifier is part of this machinery, the accumulation of *sprB* operon proteins (Figure 5.1) near the division could be due to a capture and release mechanism that requires the track to allow the *sprB* operon proteins to move to the division site.

If the polar anchor was an essential component it would not be possible to remove it and study the null mutant. Also, if it is essential, it is likely to be present in most *Bacteroidetes* as it potentially mediates multiple polar processes and not just motility. This could explain why such a component would not have been identified to date, despite the extensive transposon screening that has been done to identify gliding mutants. In fact, HubP was identified by fluorescently tagging genes that had been shown in a screen to have motility defects as a result of their deletion. Furthermore, bioinformatic approaches

that identified the important gliding motility genes by comparing motile T9SS positive strains with non-motile ones would not have identified genes that are present in both.

Alternatively, since the central gliding proteins GldDBHIJ are all lipoproteins, the polar element could also be indicated by specific lipid species that are enriched in polar regions.

Such mechanisms of polar sensing have been suggested for cardiolipin binding proteins and membrane curvature sensing protein oligomers (Surovtsev, Jacobs-Wagner, 2018).

Furthermore, in *F. johnsoniae* there are sulfonolipids which have already been shown to be important for gliding motility (Vences-Guzmán et al., 2021).

In addition to polarity, there is little knowledge about the ability of *F. johnsoniae* to navigate. Early work by Jamie Wheeler shows that *F. johnsoniae* is able to navigate spatial gradients by modulating the reversal frequency (J. H. R. Wheeler, DPhil thesis).

However, unlike chemotaxis, no specific molecule has been identified as the determinant and rather the cells sense a gradient of osmolarity (osmotaxis). When observing gliding cells with fluorescently labelled SprC in my setup, it seemed that the intensity of the SprC leading focus correlated with the frequency of reversals. The gliding cells often reversed for a brief moment before resuming motion toward the leading pole. Thus, such an event could be more or less frequent in a concentration-dependent manner, explaining such an observation. This could be part of how reversals are used to control the direction of movement.

In *M. xanthus*, the switching of the leading and lagging pole depend on an atypical variant of the methyl-accepting chemotaxis protein (MCP) system. Thus, this chemotaxis control is similar to the central paradigm in that it senses the environmental signal and initiates a

phosphorylation cascade that leads to a diffusive phosphorylated response regulator. This response regulator exerts its effect on the target protein and causes a reversal. In the case of *M. xanthus* the response regulators FtsZX diffuse to the poles and initiate release and exchange of the polar determinants and effectively causes the reversal of poles.

In *F. johnsoniae* there are no proteins with MCP signalling domains, and no mutant screen has identified MCPs or GTPases involved in gliding motility. Thus, it is likely that *F. johnsoniae* has evolved a different strategy from *M. xanthus* to control the direction of movement. I speculate that the poles are simply controlled by a catch and release of the accumulation of SprC as it moves by the pole. In this manner, a polar determinant would simply need to be informed of the energy state of the cell or other signal which would either cause the polar determinant to interact and sequester SprC or let the SprC pass by and move along towards the other pole. This, however, requires knowledge of the leading and lagging poles as the capture mechanism must be controlled to avoid sequestering SprC at both poles. Interestingly, as could be seen in chapter 4, some gliding cells had a smaller accumulation of SprC at the lagging pole. Thus, if the model of capture and release is correct, the protein(s) that allows capture resides to some extent at both ends and could be either regulated by another component to inform leading from lagging, or it could depend on the concentration at the given pole.

6.6 Future perspectives

This thesis suggests a refined version of the gliding model for *F. johnsoniae* gliding motility, and gives further clues to understanding the more general aspect of *Bacteroidetes* gliding motility. There are many elements of which we know very little.

RNA-seq data, bioinformatics, immunofluorescence microscopy and immunogold electron microscopy in fixed cells, and genetic evidence point toward GldJ as a central helical element in gliding motility. However, despite this information, there is still no methodology for visualising the protein in live cells. In this thesis, I presented the GldJ treadmilling hypothesis, which suggests that GldJ forms dynamic filaments that build from local T9SSs. Having a method for visualising GldJ *in vivo* will be key in proving or disproving that hypothesis. In order to visualise GldJ *in vivo*, a method of labelling GldJ with a fluorophore or similar must be developed. Unfortunately, several attempts at tagging GldJ suggests that it is difficult to find a spot which does not result in the post-translational degradation of GldJ. Successful tags were inserted in the C- and N-terminal ends of GldJ. Both terminal tags could not be larger than a Twin-Strep tag (~ 20 residues) without significantly reducing the spreading of the mutant strains. An attempt was also made to use fluorescein arsenical hairpin (FlAsH) labelling using the tetracysteine tag, incorporated at the unstructured region after the lipidation cysteine at the N-terminus, however, the FlAsH dye seemed to label most of the cell and the washing protocols washed out none or all of the fluorescence depending on how harsh a protocol I used. Ideally, a high-quality fluorescent marker such as the HaloTag would be successfully fused to GldJ, however if this

is not possible another option would be to optimise this tetracysteine labelling protocol in hopes that helical tracks could be observed in live cells. A similar protocol has successfully visualised flagella (Zhao et al., 2018). GldJ seem to be one large domain, making it difficult to find a region that is likely to be amenable to modifications. However, future work could also take advantage of the newly improved structure prediction tools such as alphafold (<https://alphafold.ebi.ac.uk/>), or the structure of GldJ if that is obtained, to better guide site-selection for attempts at inserting a fluorescent tag. Instead of rational engineering, another method of finding useful sites would be through transposon screening, as described in (Zordan et al., 2015). This methodology allows the random insertion of a desired tag and subsequent screening for the functional clone. Because mutants with disrupted GldJ function show as non-spreading colonies with no T9SS activity, there are multiple phenotypes which could be used to screen for stable fusions.

As an extension to the *in vivo* dynamics of GldJ, interaction with other gliding motility proteins should be an area of priority for future research. In this thesis I show that several proteins (SprC, SprD, RemG, GldD, and GldB) present in the periplasm, outer membrane, or both, are able to move in a helix. Depending on whether GldJ itself moves or not, it is important to understand how the helically moving proteins interact with GldJ. From the CXMS data, we know that they, or at least some, are in close contact with GldJ. However, we have no structural knowledge of the interaction, which in turn limits the mechanistic understanding of the system significantly. Therefore, I propose that future work should be aimed at isolating the GldJ track. Since I hypothesise that GldJ interacts with many gliding proteins (all that move helically and the T9SS

proteins GldKNO), I suspect that a successful pulldown performed without denaturing agents should present a large leap in structural knowledge of the system. Such a leap in knowledge is exemplified in the pulldown of the central secretion pore protein of the T9SS, SprA, which upon pulldown included other important T9SS proteins such as PorV and the unanticipated plug protein (Lauber et al., 2018). Attempts at purifying GldJ was done early in my project, but following issues with solubility I decided to focus more on the microscopy work. However, with the new knowledge provide here and elsewhere, I believe future structural work should indeed be possible. Specifically, a combination of *in situ* imaging using cryo electron tomography to reveal GldJ polymers in the cell, combined with single-particle reconstruction of isolated track complexes performed using a 'soft' purification protocol should provide ample knowledge on the structural aspects of this system. Potential OM track filaments has already been visualised using cryo electron tomography (Liu et al., 2007), in support of the feasibility of such an approach.

In addition to GldJ, the early results shown in this thesis suggest that the other gliding specific lipoproteins GldDBHI are also important to study further. GldD and GldB are seen to occasionally move in a helix and GldH and GldI are still to be investigated. I propose to tag GldH and GldI with HaloTag and study their movement. It would be particularly interesting to combine the dynamic movement of these proteins with that of GldJ. Furthermore, since these are lipoproteins, studies focused on the lipid composition of the membrane (e.g. sulfonolipids) could also be facilitated by studying the response of these proteins to changes in this composition. These results may well shed light on the polarity and division link with gliding motility.

It will also be interesting to understand how species without *sprB* operons control adhesive ability of their adhesins. Some species may not require this ability at all and only depend on the substrate specificity of their adhesins to get the cells to where they want to be. Alternatively, physical properties such as tug-of-war or similar ways may allow these species to glide in a defined direction without having an explicit system for controlling adhesion.

Nonetheless, the identity of leading and lagging pole must be known. Future work should be aimed at identifying the polar determinant, as this/these protein(s) likely interact with most processes that depend on polar positioning and therefore holds a wealth of information. If the polar landmark is important for multiple cellular processes that depend on the pole identity, it might be better to examine central cell process genes. Indeed, the FtsX protein is important for initiating and orchestrating cell division and a *ftsX* null mutant halts *F. johnsoniae* in a late stage of cell division. In this stage, the cells are not gliding and no helically moving SprB molecules could be identified (Kempf, McBride, 2000). Fluorescently tagging FtsX and other members of the divisome would be a good first step in identifying polar proteins involved in gliding. The knowledge of the leading pole via fluorescent SprC, as well as the dual tagging methodology that lets the user to follow two proteins in a moving cell at the same time, allows such an analysis. In addition to these divisome proteins, other cell-wall proteins such as MreB and penicillin binding proteins would also be interesting to tag and image. The small MreB inhibitor drug has already been tested on *F. johnsoniae* and was reported to have no effect (Shrivastava, Berg, 2020). However, the timeframe was rather short and if MreB is not directly involved

with gliding similar to what is seen in *M. xanthus*, then the slow orchestration of the cell wall would not have been identified. Furthermore, the cell studied in the A22 study was rotating around a fixated SprB molecule and was not responding to the cellular navigation. Thus, a simple experiment could be to add A22 to actively gliding *F. johnsoniae* cells and observe fluorescently labelled gliding proteins.

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Chapter 7

Appendix

7.1 Table of strains

Table 7.1: List of strains created during my DPhil studies.

Strain	Strain Description	Parent Strain
sAK0019	<i>F. johnsoniae</i> UW101 <i>gldJ</i> ::Asp202:: <i>halotag</i>	<i>F. johnsoniae</i> UW101
sAK0025	<i>F. johnsoniae</i> UW101 <i>gldJ</i> ::Gly462:: <i>halotag</i>	<i>F. johnsoniae</i> UW101
sAK0027	<i>F. johnsoniae</i> UW101 <i>gldJ</i> ::Lys309:: <i>halotag</i>	<i>F. johnsoniae</i> UW101
sAK0028	<i>F. johnsoniae</i> UW101 <i>gldJ</i> ::Glu123:: <i>halotag</i>	<i>F. johnsoniae</i> UW101
sAK0029	<i>F. johnsoniae</i> UW101 <i>gldJ</i> ::Val247:: <i>halotag</i>	<i>F. johnsoniae</i> UW101
sAK0036	<i>F. johnsoniae</i> UW101 <i>gldJ</i> -Ct-TS	<i>F. johnsoniae</i> UW101
sAK0043	<i>F. johnsoniae</i> UW101 SP- <i>sprB</i> - <i>twin-strep-tag</i> :: <i>halotag-sprB</i>	<i>F. johnsoniae</i> UW101

Strain	Strain Description	Parent Strain
sAK0045	<i>F. johnsoniae</i> UW101 SP- <i>sprB</i> -twin-strep-tag:: <i>halotag-sprB</i> -448	<i>F. johnsoniae</i> UW101
sAK0049	<i>F. johnsoniae</i> UW101 <i>gldJ</i> -N-term-Twin-Strep	<i>F. johnsoniae</i> UW101
sAK0053	<i>F. johnsoniae</i> UW101 <i>sprC</i> N-term-TS	<i>F. johnsoniae</i> UW101
sAK0057	<i>F. johnsoniae</i> UW101 SP- <i>sprB</i> -twin-strep-tag:: <i>halotag-sprB</i> -218	<i>F. johnsoniae</i> UW101
sAK0061	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag-sprB</i> -218	BB259 (FL030)
sAK0062	<i>F. johnsoniae</i> UW101 Δ <i>sprA</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag-sprB</i> -218	BB233 (FL004)
sAK0063	<i>F. johnsoniae</i> UW101 Δ <i>porU</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag-sprB</i> -218	BB367 (FL094)
sAK0067	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag-sprB</i> -448	BB259 (FL030)
sAK0068	<i>F. johnsoniae</i> UW101 Δ <i>sprA</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag-sprB</i> -448	BB233 (FL004)
sAK0069	<i>F. johnsoniae</i> UW101 Δ <i>porU</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag-sprB</i> -448	BB367 (FL094)

Strain	Strain Description	Parent Strain
sAK0073	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i>	BB259 (FL030)
sAK0074	<i>F. johnsoniae</i> UW101 Δ_{sprA} SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i>	BB233 (FL004)
sAK0075	<i>F. johnsoniae</i> UW101 Δ_{porU} SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i>	BB367 (FL094)
sAK0079	<i>F. johnsoniae</i> UW101 <i>sprD</i> -N-term-TS (singlestrep)	<i>F. johnsoniae</i> UW101
sAK0108	<i>F. johnsoniae</i> UW101 SP- <i>sprD</i> -6xHis- <i>sprD</i>	<i>F. johnsoniae</i> UW101
sAK0109	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> -6xHis- <i>sprC</i>	<i>F. johnsoniae</i> UW101
sAK0112	<i>F. johnsoniae</i> UW101 SP- <i>sprD</i> -mCherry- <i>sprD</i>	<i>F. johnsoniae</i> UW101
sAK0113	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> -mCherry- <i>sprC</i>	<i>F. johnsoniae</i> UW101
sAK0123	<i>F. johnsoniae</i> UW101 <i>gldJ72CTD</i>	<i>F. johnsoniae</i> UW101
sAK0124	<i>F. johnsoniae</i> UW101 <i>gldJ72CTD</i> -C-term-6xHis	<i>F. johnsoniae</i> UW101
sAK0125	<i>F. johnsoniae</i> UW101 <i>gldJ72CTD</i> -C-term-twin-strep	<i>F. johnsoniae</i> UW101
sAK0126	<i>F. johnsoniae</i> UW101 <i>gldJ553</i>	<i>F. johnsoniae</i> UW101
sAK0127	<i>F. johnsoniae</i> UW101 <i>gldJ553</i> -N-term-6xHis	<i>F. johnsoniae</i> UW101
sAK0128	<i>F. johnsoniae</i> UW101 <i>gldJ553</i> -N-term-twin-strep	<i>F. johnsoniae</i> UW101

Strain	Strain Description	Parent Strain
sAK0129	<i>F. johnsoniae</i> UW101 <i>gldJ</i> 548	<i>F. johnsoniae</i> UW101
sAK0130	<i>F. johnsoniae</i> UW101 <i>gldJ</i> 548-N-term-6xHis	<i>F. johnsoniae</i> UW101
sAK0131	<i>F. johnsoniae</i> UW101 <i>gldJ</i> 548-N-term-twin-strep	<i>F. johnsoniae</i> UW101
sAK0139	<i>F. johnsoniae</i> UW101 <i>gldJ</i> -N-term-6xHis	<i>F. johnsoniae</i> UW101
sAK0141	<i>F. johnsoniae</i> UW101 <i>gldJ</i> -N-term-mCherry	<i>F. johnsoniae</i> UW101
sAK0142	<i>F. johnsoniae</i> UW101 $\Delta porV$ SP- <i>sprB</i> -twin-strep-tag::halotag- <i>sprB</i> -448 <i>sprC</i> -nt-mCherry	SAK67
sAK0149	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> -twin-strep-tag::halotag- <i>sprC</i>	<i>F. johnsoniae</i> UW101
sAK0151	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> -snap-tag- <i>sprC</i>	<i>F. johnsoniae</i> UW101
sAK0153	<i>F. johnsoniae</i> UW101 $\Delta porV$ SP- <i>sprC</i> -twin-strep-tag::halotag- <i>sprC</i>	BB259 (FL030)
sAK0155	<i>F. johnsoniae</i> UW101 $\Delta porV$ SP- <i>sprC</i> -snap-tag- <i>sprC</i>	BB259 (FL030)
sAK0157	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> -twin-strep-tag::halotag- <i>sprC</i> <i>gldJ</i> 553	sAK0126
sAK0159	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> -snap-tag- <i>sprC</i> <i>gldJ</i> 553	sAK0126

Strain	Strain Description	Parent Strain
sAK0161	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> - <i>twin-strep-tag::halotag-sprC</i> <i>gldJ</i> 548	sAK0129
sAK0163	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> - <i>snap-tag-sprC</i> <i>gldJ</i> 548	sAK0129
sAK0165	<i>F. johnsoniae</i> UW101 Δ <i>gldL</i> Δ <i>porV</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448	sAK0067
sAK0167	<i>F. johnsoniae</i> UW101 Δ <i>gldM</i> Δ <i>porV</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448	sAK0067
sAK0170	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> <i>gldJ</i> 553-N-term-6xHis	BB259 (FL030)
sAK0171	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> <i>gldJ</i> 553-N-term- <i>twin-strep</i>	BB259 (FL030)
sAK0184	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448 <i>gldL</i> -Y13A	sAK0165
sAK0185	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448 <i>gldL</i> -H30A	sAK0165
sAK0186	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448 <i>gldL</i> -K27A	sAK0165

Strain	Strain Description	Parent Strain
sAK0187	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448 <i>gldM</i> -Y17F	sAK0167
sAK0188	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448 <i>gldJ</i> 553	sAK0067
sAK0189	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448 <i>gldJ</i> 548	sAK0067
sAK0196	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> <i>gldL</i> -Y13A	sAK0205
sAK0197	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> <i>gldL</i> -H30A	sAK0205
sAK0198	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> <i>gldL</i> -K27A	sAK0205
sAK0199	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> <i>gldM</i> -Y17F	sAK0203
sAK0200	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> <i>gldJ</i> 553	SAK0073
sAK0201	<i>F. johnsoniae</i> UW101 Δ_{porV} SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> <i>gldJ</i> 548	SAK0073

Strain	Strain Description	Parent Strain
sAK0203	<i>F. johnsoniae</i> UW101 $\Delta gldM$ $\Delta porV$ SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i>	SAK0073
sAK0205	<i>F. johnsoniae</i> UW101 $\Delta gldL$ $\Delta porV$ SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i>	SAK0073
sAK0208	<i>F. johnsoniae</i> UW101 $\Delta sprF$ SP- <i>sprC</i> - <i>twin</i> - <i>strep-tag::halotag-sprC</i>	BB397 (FL115)
sAK0210	<i>F. johnsoniae</i> UW101 $\Delta sprB$ SP- <i>sprC</i> - <i>twin</i> - <i>strep-tag::halotag-sprC</i>	BB302 (McBride)
sAK0212	<i>F. johnsoniae</i> UW101 $\Delta sprA$ SP- <i>sprC</i> - <i>twin</i> - <i>strep-tag::halotag-sprC</i>	BB233 (FL004)
sAK0214	<i>F. johnsoniae</i> UW101 $\Delta gldJ$ SP- <i>sprC</i> - <i>twin-strep</i> - <i>tag::halotag-sprC</i>	BB215
sAK0241	<i>E. coli</i> BL21 pET15b- <i>sprC</i>	-
sAK0253	<i>E. coli</i> BL21 pET15b- <i>gldJ</i> 45-410	-
sAK0254	<i>E. coli</i> BL21 PET15b- <i>gldJ</i> 470-561	-
sAK0271	<i>F. johnsoniae</i> UW101 <i>sprD</i> -C-term-Twin-Strep	<i>F. johnsoniae</i> UW101
sAK0272	<i>F. johnsoniae</i> UW101 <i>sprD</i> -C-term-singleStrep	<i>F. johnsoniae</i> UW101
sAK0273	<i>F. johnsoniae</i> UW101 <i>sprD</i> -C-term-6xHis	<i>F. johnsoniae</i> UW101
sAK0274	<i>F. johnsoniae</i> UW101 <i>gldJ</i> -N-term-TC	<i>F. johnsoniae</i> UW101

Strain	Strain Description	Parent Strain
sAK0275	<i>F. johnsoniae</i> UW101 <i>gldJ</i> -C-term-TC	<i>F. johnsoniae</i> UW101
sAK0276	<i>F. johnsoniae</i> UW101 Δ <i>sprC</i>	<i>F. johnsoniae</i> UW101
sAK0277	<i>F. johnsoniae</i> UW101 Δ <i>gldK</i> SP- <i>sprC</i> - <i>twin-strep-tag::halotag-sprC</i>	SAK0149
sAK0278	<i>F. johnsoniae</i> UW101 Δ <i>gldKLMNO</i> SP- <i>sprC</i> - <i>twin-strep-tag::halotag-sprC</i>	SAK0149
sAK0279	<i>F. johnsoniae</i> UW101 Δ <i>gldKLMN</i>	<i>F. johnsoniae</i> UW101
sAK0280	<i>F. johnsoniae</i> UW101 Δ <i>gldNO</i>	<i>F. johnsoniae</i> UW101
sAK0281	<i>F. johnsoniae</i> UW101 Δ <i>fjoh1517</i>	<i>F. johnsoniae</i> UW101
sAK0282	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2422</i>	<i>F. johnsoniae</i> UW101
sAK0283	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2733</i>	<i>F. johnsoniae</i> UW101
sAK0284	<i>F. johnsoniae</i> UW101 Δ <i>fjoh5040</i>	<i>F. johnsoniae</i> UW101
sAK0285	<i>F. johnsoniae</i> UW101 Δ <i>remA</i>	<i>F. johnsoniae</i> UW101
sAK0286	<i>F. johnsoniae</i> UW101 Δ <i>remC</i>	<i>F. johnsoniae</i> UW101
sAK0289	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> <i>gldMR9K</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i>	sAK0203
sAK0291	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> Δ <i>sprC</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448	sAK0067

Strain	Strain Description	Parent Strain
sAK0293	<i>F. johnsoniae</i> UW101 Δ <i>fjoh5040</i> SP- <i>sprB</i> -twin- <i>strep-tag::halotag-sprB</i> -448 Δ <i>porV</i>	sAK0067
sAK0295	<i>F. johnsoniae</i> UW101 Δ <i>fjoh5040</i> SP- <i>sprC</i> -twin- <i>strep-tag::halotag-sprC</i>	SAK0149
sAK0297	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> SP- <i>sprC</i> - <i>snap-tag</i> - <i>sprC</i> SP- <i>sprB</i> -twin- <i>strep-tag::halotag-sprB</i> -448	sAK0067
sAK0299	<i>F. johnsoniae</i> UW101 Δ <i>gldL</i> SP- <i>sprC</i> -twin- <i>strep</i> - <i>tag::halotag-sprC</i>	SAK0149
sAK0301	<i>F. johnsoniae</i> UW101 Δ <i>gldM</i> SP- <i>sprC</i> -twin- <i>strep-tag::halotag-sprC</i>	SAK0149
sAK0347	<i>F. johnsoniae</i> UW101 Δ <i>SprE</i> Δ <i>porV</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448	sAK0067
sAK0348	<i>F. johnsoniae</i> UW101 Δ <i>SprE</i> SP- <i>sprC</i> -twin- <i>strep-tag::halotag-sprC</i>	SAK0149
sAK0349	<i>F. johnsoniae</i> <i>sprA::halotag</i> SP- <i>sprC</i> - <i>snap-tag</i> - <i>sprC</i>	SAK151
sAK0350	<i>F. johnsoniae</i> UW101 <i>sprA-snap</i> SP- <i>sprB</i> -twin- <i>strep-tag::halotag-sprB</i> -448 Δ <i>porV</i>	sAK0067

Strain	Strain Description	Parent Strain
sAK0351	<i>F. johnsoniae</i> UW101 <i>sprA-snap</i> SP- <i>sprC-twin-strep-tag::halotag-sprC</i>	SAK0149
sAK0352	<i>F. johnsoniae</i> SprE:: <i>halotag</i> SP- <i>sprC-snap-tag-sprC</i>	SAK0151
sAK0353	<i>F. johnsoniae</i> UW101 Δ <i>sprA</i> -N-term Δ <i>porV</i> SP- <i>sprB-twin-strep-tag::halotag-sprB-448</i>	sAK0067
sAK0354	<i>F. johnsoniae</i> UW101 Δ <i>sprA</i> -N-term Δ <i>porV</i> SP- <i>sprB-twin-strep-tag::halotag-sprB</i>	SAK0073
sAK0355	<i>F. johnsoniae</i> UW101 Δ <i>sprA</i> -N-term SP- <i>sprC-twin-strep-tag::halotag-sprC</i>	SAK0149
sAK0356	<i>F. johnsoniae</i> UW101 <i>fjoh5040-ct::halotag</i>	<i>F. johnsoniae</i> UW101
sAK0357	<i>F. johnsoniae</i> UW101 <i>fjoh5040-ct::halotag</i> SP- <i>sprC-snap-tag-sprC</i>	SAK0151
sAK0358	<i>F. johnsoniae</i> UW101 Δ <i>sprD</i>	<i>F. johnsoniae</i> UW101
sAK0359	<i>F. johnsoniae</i> UW101 Δ <i>sprC</i>	<i>F. johnsoniae</i> UW101
sAK0360	<i>F. johnsoniae</i> UW101 Δ <i>sprC</i> Δ <i>porV</i> SP- <i>sprB-twin-strep-tag::halotag-sprB</i>	SAK0073
sAK0361	<i>F. johnsoniae</i> UW101 Δ <i>sprD</i> Δ <i>porV</i> SP- <i>sprB-twin-strep-tag::halotag-sprB</i>	SAK0073

Strain	Strain Description	Parent Strain
sAK0362	<i>F. johnsoniae</i> UW101 $\Delta gldNO$ SP- <i>sprC</i> - <i>twin-strep-tag::halotag-sprC</i>	SAK0149
sAK0363	<i>F. johnsoniae</i> UW101 $\Delta gldO$ SP- <i>sprC</i> - <i>twin-strep-tag::halotag-sprC</i>	SAK0149
sAK0365	<i>F. johnsoniae</i> $\Delta gldK$ <i>gldJ</i> -nt-TS	SAK49
sAK0366	<i>F. johnsoniae</i> $\Delta gldK$ <i>gldJ</i> -nt-TC	SAK0274
sAK0367	<i>F. johnsoniae</i> $\Delta gldK$ <i>gldJ</i> -ct-TC	SAK0275
sAK0368	<i>F. johnsoniae</i> UW101 <i>remA</i> -nt:: <i>halotag</i>	<i>F. johnsoniae</i> UW101
sAK0369	<i>F. johnsoniae</i> UW101 <i>remA</i> -nt:: <i>halotag</i> SP- <i>sprC</i> - <i>snap-tag-sprC</i>	SAK151
sAK0377	<i>F. johnsoniae</i> UW101 $\Delta fjoh5040$ SP- <i>sprC</i> - <i>snap-tag-sprC</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448 $\Delta porV$	SAK0297
sAK0378	<i>F. johnsoniae</i> UW101 <i>remA</i> -nt:: <i>halotag</i> SP- <i>sprC</i> - <i>snap-tag-sprC</i> SP- <i>sprB</i> - <i>twin-strep-tag::halotag-sprB</i> -448 $\Delta porV$	SAK0297
sAK0385	<i>F. johnsoniae</i> UW101 $\Delta tssC$	sAK0010
sAK0387	<i>F. johnsoniae</i> UW101 $\Delta tssC$ <i>gldJ</i> 548	sAK0129
sAK0389	<i>F. johnsoniae</i> UW101 $\Delta tssC$ $\Delta sprA$	BB0233

Strain	Strain Description	Parent Strain
sAK0391	<i>F. johnsoniae</i> UW101 $\Delta tssC$ $\Delta gldJ$	BB0215
sAK0393	<i>F. johnsoniae</i> UW101 $\Delta tssC$ $\Delta porV$	BB0259
sAK0395	<i>F. johnsoniae</i> UW101 $\Delta tssC$ $\Delta sprF$	BB0397
sAK0397	<i>F. johnsoniae</i> UW101 $\Delta porV$ SP- <i>sprB</i> -Twin- Strep:: <i>halotag-sprB</i> SP- <i>sprC</i> -snap-tag- <i>sprC</i>	sAK0073
sAK0399	<i>F. johnsoniae</i> UW101 $\Delta sprD$ SP- <i>sprC</i> -snap-tag- <i>sprC</i>	sAK0151
sAK0401	<i>F. johnsoniae</i> UW101 $\Delta sprD$ SP- <i>sprC</i> -twin- strep-tag:: <i>halotag-sprC</i>	sAK0149
sAK0403	<i>F. johnsoniae</i> UW101 $\Delta sprD$ SP- <i>sprC</i> -twin- strep-tag:: <i>halotag-sprC</i> $\Delta sprF$	sAK0208
sAK0408	<i>E. coli</i> BL21 PET15b- <i>sprD</i> -ct-200aa	-
sAK0409	<i>E. coli</i> BL21 PET15b- <i>fjoh5040</i>	-
sAK0412	<i>F. johnsoniae</i> UW101 SP- <i>sprC</i> -twin-strep- tag:: <i>halotag-sprC</i> <i>gldLE49Q</i>	sAK299
sAK0416	<i>E. coli</i> BL21 PET15b- <i>sprD</i> -Soluble domain	-
sAK0417	<i>E. coli</i> BL21 PET15b- <i>sprD</i> -ct-1154-end	-
sAK0429	<i>F. johnsoniae</i> UW101 $\Delta fjoh0798$ $\Delta porV$ SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i>	sAK0073

Strain	Strain Description	Parent Strain
sAK0430	<i>F. johnsoniae</i> UW101 Δ <i>fjoh0798</i> Δ <i>porV</i> SP- <i>sprB-twin-strep-tag::halotag-sprB</i> -448	SAK0067
sAK0431	<i>F. johnsoniae</i> UW101 Δ <i>fjoh0798</i> SP- <i>sprB-twin-</i> <i>strep-tag::halotag-sprB</i>	sAK0043
sAK0432	<i>F. johnsoniae</i> UW101 Δ <i>fjoh0798</i> SP- <i>sprB-twin-</i> <i>strep-tag::halotag-sprB</i> -448	sAK0045
sAK0433	<i>F. johnsoniae</i> UW101 Δ <i>fjoh0798</i>	sAK0010
sAK0434	<i>F. johnsoniae</i> UW101 <i>sprD</i> -6xHis SP- <i>sprC-twin-</i> <i>strep-tag::halotag-sprC</i>	SAK0149
sAK0437	<i>E. coli</i> Rosetta PET15b- <i>sprD</i> -Soluble domain	-
sAK0438	<i>E. coli</i> Rosetta pET15b- <i>gldJ</i> 45-410	-
sAK0439	<i>E. coli</i> Rosetta PET15b- <i>gldJ</i> 470-561	-
sAK0440	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA-</i> <i>sprD</i> _1154-1588 SP- <i>sprC-twin-strep-tag::halotag-</i> <i>sprC</i>	SAK0149
sAK0441	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA-sprD</i> _1154-1588 Δ <i>porV</i> SP- <i>sprB</i> -Twin- Strep:: <i>halotag-sprB</i>	sAK0073

Strain	Strain Description	Parent Strain
sAK0444	<i>F. johnsoniae</i> UW101 <i>sprD</i> _1538 SP- <i>sprC</i> -twin- <i>strep-tag::halotag-sprC</i>	SAK0149
sAK0446	<i>F. johnsoniae</i> UW101 <i>sprD</i> _1538	SAK0010
sAK0447	<i>F. johnsoniae</i> UW101 pCP11-PompA-SP- <i>ompA</i> - <i>sprD</i> _1154-1588 Δ <i>sprD</i> SP- <i>sprC</i> -twin- <i>strep</i> - <i>tag::halotag-sprC</i>	sAK0401
sAK0451	<i>F. johnsoniae</i> UW101 <i>sprD</i> _1538 Δ <i>fjoh0798</i>	sAK0433
sAK0452	<i>F. johnsoniae</i> UW101 <i>sprD</i> _1538 SP- <i>sprB</i> -twin- <i>strep-tag::halotag-sprB</i>	sAK0043
sAK0453	<i>F. johnsoniae</i> UW101 <i>sprD</i> _1538 Δ <i>fjoh0798</i> SP- <i>sprB</i> -twin- <i>strep-tag::halotag-sprB</i>	sAK0431
sAK0456	<i>F. johnsoniae</i> UW101 pCP11- <i>gldJ</i> Δ <i>porV</i> SP- <i>sprB</i> -Twin-Strep::halotag- <i>sprB</i>	sAK0073
sAK0457	<i>F. johnsoniae</i> UW101 pCP11- <i>gldJ</i> Δ <i>porV</i> SP- <i>sprB</i> -twin- <i>strep-tag::halotag-sprB</i> -448	SAK0067
sAK0458	<i>F. johnsoniae</i> UW101 pCP11- <i>gldJ</i> SP- <i>sprC</i> - <i>snap-tag-sprC</i> SP- <i>sprB</i> -twin- <i>strep-tag::halotag</i> - <i>sprB</i> -448 Δ <i>porV</i>	SAK0297

Strain	Strain Description	Parent Strain
sAK0459	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA</i> - <i>sprD</i> _1154-1588 SP- <i>sprC</i> - <i>snap</i> -tag- <i>sprC</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag</i> - <i>sprB</i> -448 Δ <i>porV</i>	SAK0297
sAK0462	<i>F. johnsoniae</i> UW101 Δ <i>sprCD</i> Δ <i>porV</i> SP- <i>sprB</i> - Twin-Strep:: <i>halotag</i> - <i>sprB</i>	sAK0073
sAK0465	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA</i> - <i>sprD</i> _1154-1588 SP- <i>sprC</i> -twin-strep-tag:: <i>halotag</i> - <i>sprC</i> <i>gldJ</i> 548	sAK0161
sAK0466	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA</i> - <i>sprD</i> _1154-1588 Δ <i>porV</i> SP- <i>sprB</i> -twin-strep- tag:: <i>halotag</i> - <i>sprB</i> -448 <i>gldJ</i> 548	sAK0189
sAK0467	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA</i> - <i>sprD</i> _1154-1588 Δ <i>porV</i> SP- <i>sprB</i> -twin-strep- tag:: <i>halotag</i> - <i>sprB</i> <i>gldJ</i> 548	sAK0201
sAK0468	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA</i> - <i>sprD</i> _1154-1588 Δ <i>gldKLMNO</i> SP- <i>sprC</i> -twin- strep-tag:: <i>halotag</i> - <i>sprC</i>	sAK0278

Strain	Strain Description	Parent Strain
sAK0469	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA</i> - <i>sprD</i> _1154-1588 Δ <i>sprD</i> Δ <i>porV</i> SP- <i>sprB</i> -twin- <i>strep-tag::halotag-sprB</i>	sAK0361
sAK0471	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA</i> - <i>sprD</i> _1154-1588 Δ <i>sprCD</i> Δ <i>porV</i> SP- <i>sprB</i> -Twin- Strep:: <i>halotag-sprB</i>	sAK0462
sAK0474	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA-sprD</i> _1154-1588 Δ <i>porV</i> SP- <i>sprB</i> -Twin- Strep:: <i>halotag-sprB</i> SP- <i>sprC-snap-tag-sprC</i>	sAK0397
sAK0475	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA</i> -SP- <i>ompA</i> - <i>sprD</i> _1154-1588 Δ <i>gldJ</i> SP- <i>sprC-twin-strep</i> - <i>tag::halotag-sprC</i>	sAK0214
sAK0482	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA-sprF</i> -nt- 8xHis Δ <i>porV</i> SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i> SP- <i>sprC-snap-tag-sprC</i>	sAK0397
sAK0483	<i>F. johnsoniae</i> UW101 pCP11- <i>PompA-sprF</i> -ct- 8xHis Δ <i>porV</i> SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i> SP- <i>sprC-snap-tag-sprC</i>	sAK0397

Strain	Strain Description	Parent Strain
sAK0484	<i>F. johnsoniae</i> UW101 pCP11-PompA-SP-ompA- <i>fjoh5040</i> Δ <i>porV</i> SP- <i>sprB</i> -Twin-Strep:: <i>halotag</i> - <i>sprB</i> SP- <i>sprC</i> -snap-tag- <i>sprC</i>	sAK0397
sAK0493	<i>F. johnsoniae</i> UW101 <i>sprD</i> :: <i>halotag</i>	sAK0010
sAK0494	<i>F. johnsoniae</i> UW101 <i>sprD</i> :: <i>halotag</i> SP- <i>sprC</i> - <i>snap</i> -tag- <i>sprC</i>	sAK0151
sAK0495	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2389</i> Δ <i>fjoh0798</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag</i> - <i>sprB</i>	sAK0431
sAK0496	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2389</i> Δ <i>fjoh0798</i> SP- <i>sprB</i> -twin-strep-tag:: <i>halotag</i> - <i>sprB</i> -448	sAK0432
sAK0497	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2389</i> Δ <i>fjoh0798</i>	sAK0433
sAK0498	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2389</i> SP- <i>sprB</i> -twin- <i>strep</i> -tag:: <i>halotag</i> - <i>sprB</i>	sAK0043
sAK0499	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2389</i> SP- <i>sprB</i> -twin- <i>strep</i> -tag:: <i>halotag</i> - <i>sprB</i> -448	sAK0045
sAK0500	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2389</i>	sAK0010
sAK0524	<i>F. johnsoniae</i> UW101 <i>gldJ548</i> Δ <i>porV</i> SP- <i>sprB</i> - Twin-Strep:: <i>halotag</i> - <i>sprB</i> SP- <i>sprC</i> -snap-tag- <i>sprC</i>	sAK0397

Strain	Strain Description	Parent Strain
sAK0525	<i>F. johnsoniae</i> UW101 pCP11-PompA-SP- <i>ompA-sprD</i> _1154-1538 Δ <i>porV</i> SP- <i>sprB</i> -Twin- Strep:: <i>halotag-sprB</i> SP- <i>sprC</i> -snap-tag- <i>sprC</i>	sAK0397
sAK0536	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> SP- <i>sprB</i> -6xHis- <i>sprB</i> -448	sAK0073
sAK0537	<i>F. johnsoniae</i> UW101 Δ <i>fjoh1645-1647</i>	sAK0010
sAK0538	<i>F. johnsoniae</i> UW101 Δ <i>fjoh1677</i>	sAK0010
sAK0539	<i>F. johnsoniae</i> UW101 Δ <i>fjoh1985-1986</i>	sAK0010
sAK0540	<i>F. johnsoniae</i> UW101 Δ <i>fjoh2274-2275</i>	sAK0010
sAK0541	<i>F. johnsoniae</i> UW101 Δ <i>fjoh3476-3478</i>	sAK0010
sAK0542	<i>F. johnsoniae</i> UW101 Δ <i>fjoh3950-3952</i>	sAK0010
sAK0543	<i>F. johnsoniae</i> UW101 Δ <i>fjoh3971-3973</i>	sAK0010
sAK0544	<i>F. johnsoniae</i> UW101 Δ <i>fjoh4538-4540</i>	sAK0010
sAK0545	<i>F. johnsoniae</i> UW101 Δ <i>fjoh4749-4750</i>	sAK0010
sAK0548	<i>F. johnsoniae</i> UW101 Δ <i>remG</i> Δ <i>remI</i> SP- <i>sprC</i> - <i>twin-strep-tag::halotag-sprC</i>	sZS109
sAK0549	<i>F. johnsoniae</i> UW101 Δ <i>remG</i> Δ <i>remI</i> <i>sprD::halotag</i>	sZS109

Strain	Strain Description	Parent Strain
sAK0558	<i>F. johnsoniae</i> UW101 <i>sprD</i> -1-633 SP- <i>sprC</i> -twin- <i>strep-tag::halotag-sprC</i>	SAK0149
sAK0559	<i>F. johnsoniae</i> UW101 <i>sprD</i> -1-633 <i>remG::halotag</i>	sZS121
sAK0560	<i>F. johnsoniae</i> UW101 <i>remG</i> -Rigid-linker:: <i>halotag</i>	sAK0010
sAK0562	<i>F. johnsoniae</i> UW101 $\Delta porV$ <i>sprD</i> 1-1538 SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i>	sAK0073
sAK0564	<i>F. johnsoniae</i> UW101 <i>sprD</i> :: <i>halotag</i> $\Delta gldKLMNO$	sAK0493
sAK0566	<i>F. johnsoniae</i> UW101 <i>sprD</i> :: <i>halotag</i> <i>gldJ</i> 548	sAK0493
sAK0568	<i>F. johnsoniae</i> UW101 <i>sprD</i> :: <i>halotag</i> $\Delta sprA$ -Nt	sAK0493
sAK0570	<i>F. johnsoniae</i> UW101 <i>sprD</i> :: <i>halotag</i> <i>gldJ</i> 548	sAK0129
sAK0572	<i>F. johnsoniae</i> UW101 <i>sprD</i> :: <i>halotag</i> $\Delta gldJ$	BB215
sAK0574	<i>F. johnsoniae</i> UW101 <i>sprD</i> :: <i>halotag</i> $\Delta sprA$	BB233
sAK0576	<i>F. johnsoniae</i> UW101 $\Delta porV$ $\Delta remG$ $\Delta remI$	sZS109
sAK0578	<i>F. johnsoniae</i> UW101 $\Delta remF$	sAK0010
sAK0580	<i>F. johnsoniae</i> UW101 $\Delta remH$ $\Delta remFG$	sZS77
sAK0582	<i>F. johnsoniae</i> UW101 $\Delta remF$ $\Delta remH$	sZS60
sAK0586	<i>F. johnsoniae</i> UW101 $\Delta porV$ $\Delta remF$ SP- <i>sprB</i> - Twin-Strep:: <i>halotag-sprB</i>	sAK0073

Strain	Strain Description	Parent Strain
sAK0588	<i>F. johnsoniae</i> UW101 $\Delta porV$ $\Delta remH$ SP- <i>sprB</i> - Twin-Strep:: <i>halotag-sprB</i>	sAK0073
sAK0592	<i>F. johnsoniae</i> UW101 <i>sprD</i> -1-309 SP- <i>sprC</i> - <i>twin</i> - <i>strep-tag</i> :: <i>halotag-sprC</i>	SAK0149
sAK0593	<i>F. johnsoniae</i> UW101 <i>sprD</i> -1-309 <i>remG</i> :: <i>halotag</i>	sAK0559
sAK0595	<i>F. johnsoniae</i> UW101 $\Delta porV$ $\Delta remFH$ SP- <i>sprB</i> - Twin-Strep:: <i>halotag-sprB</i>	sAK0588
sAK0597	<i>F. johnsoniae</i> UW101 $\Delta porV$ $\Delta remFH$ SP- <i>sprB</i> - Twin-Strep:: <i>halotag-sprB</i>	sAK0586
sAK0599	<i>F. johnsoniae</i> UW101 <i>sprD</i> -1-309 <i>remG</i> :: <i>halotag</i>	sZS121
sAK0601	<i>F. johnsoniae</i> UW101 <i>sprD</i> -1-309	sAK0010
sAK0603	<i>F. johnsoniae</i> UW101 $\Delta porV$ $\Delta remG$ $\Delta remI$ SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i>	sAK0606
sAK0606	<i>F. johnsoniae</i> UW101 $\Delta porV$ $\Delta remG$ SP- <i>sprB</i> - Twin-Strep:: <i>halotag-sprB</i>	sAK0073
sAK0616	<i>F. johnsoniae</i> UW101 <i>gldB</i> :: <i>halotag</i>	sAK0010
sAK0617	<i>F. johnsoniae</i> UW101 <i>gldD</i> :: <i>halotag</i>	sAK0010
sAK0618	<i>F. johnsoniae</i> UW101 <i>gldB</i> :: <i>halotag</i> SP- <i>sprC</i> - <i>snap-tag-sprC</i>	sAK0151

Strain	Strain Description	Parent Strain
sAK0619	<i>F. johnsoniae</i> UW101 <i>gldD::halotag</i> SP- <i>sprC</i> - <i>snap-tag-sprC</i>	sAK0151
sAK0621	<i>F. johnsoniae</i> UW101 Δ <i>sprC</i> <i>sprD::halotag</i>	sAK0493
sAK0625	<i>F. johnsoniae</i> UW101 Δ <i>sprF</i> <i>sprD::halotag</i>	BB397 (FL115)
sAK0627	<i>F. johnsoniae</i> UW101 Δ <i>sprF</i> <i>remG::halotag</i>	BB397 (FL115)
sAK0633	<i>F. johnsoniae</i> UW101 Δ <i>porV</i> <i>sprD</i> _1538 SP- <i>sprB</i> -Twin-Strep:: <i>halotag-sprB</i>	NA
sAK0635	<i>F. johnsoniae</i> UW101 <i>sprD</i> -1-633:: <i>halotag</i>	sAK0010
sAK0637	<i>F. johnsoniae</i> UW101 <i>sprD</i> -1-309:: <i>halotag</i>	

7.2 List of Plasmids

Table 7.2: Plasmids created during my DPhil project.

Plasmid.ID	Description	Origin
pAK0015	Used as template to amplify <i>halotag::twinstrep</i>	This Study
pAK0016	Suicide plasmid used to introduce <i>twin</i> - <i>strep::halotag::sprB</i> _{CTD218}	This Study

Plasmid.ID	Description	Origin
pAK0017	Suicide plasmid used to introduce <i>twin-strep::halotag::sprB_{CTD448}</i>	This Study
pAK0021	Suicide plasmid used to introduce <i>twin-strep::halotag::sprB</i>	This Study
pAK0001	Used as template for the construction of pAK0028 and pAK031	This Study
pAK0028	Suicide plasmid used to introduce <i>gldJ553</i>	This Study
pAK0031	Suicide plasmid used to introduce <i>gldJ548</i>	This Study
pAK0040	Suicide plasmid used to introduce <i>twin-strep::halotag::sprC</i>	This Study
pAK0041	Suicide plasmid used to introduce <i>snaptag::sprC</i>	This Study
pAK0042	Used as template for the construction of pAK0081, pAK0087, pAK0088, and pAK0089	This Study
pAK0048	Suicide plasmid used to delete <i>sprC</i>	This Study
pAK0050	Suicide plasmid used to delete <i>gldK</i>	This Study
pAK0051	Suicide plasmid used to delete <i>gldKLMNO</i>	This Study
pAK0053	Suicide plasmid used to delete <i>gldNO</i>	This Study
pAK0054	Suicide plasmid used to delete <i>gldO</i>	This Study

Plasmid.ID	Description	Origin
pAK0055	Suicide plasmid used to delete <i>fjoh1517</i>	This Study
pAK0056	Suicide plasmid used to delete <i>fjoh2422</i>	This Study
pAK0057	Suicide plasmid used to delete <i>fjoh2733</i>	This Study
pAK0058	Suicide plasmid used to delete <i>fjoh5040</i>	This Study
pAK0064	Suicide plasmid used to delete <i>sprD</i> in the background of <i>twinstrep::halotag::sprB</i>	This Study
pAK0068	Suicide plasmid used to delete <i>sprD</i> in UW101	This Study
pAK0069	Suicide plasmid used to introduce <i>fjoh5040</i> -C-term:: <i>halotag</i>	This Study
pAK0076	Suicide plasmid used to delete <i>sprCD</i> in the background of <i>twinstrep::halotag::sprB</i>	This Study
pAK0081	Suicide plasmid used to introduce <i>sprD</i> ₁₅₃₈	This Study
pAK0082	Replicative plasmid used to overexpress <i>sprD</i> _{1154–1588} from an <i>ompA</i> promoter	This Study
pAK0087	Suicide plasmid used to introduce <i>sprD::halotag</i>	This Study
pAK0088	Replicative plasmid used to overexpress <i>fjoh5040</i> from an <i>ompA</i> promoter	This Study

Plasmid.ID	Description	Origin
pAK0089	Replicative plasmid used to overexpress <i>sprD</i> _{1154–1538} from an <i>ompA</i> promoter	This Study
pAK0090	Suicide plasmid used to insert 6xHis at the N-terminus of <i>sprB</i>	This Study
pAK0091	Suicide plasmid used to delete <i>fjoh1645</i> -1647	This Study
pAK0092	Suicide plasmid used to delete <i>fjoh1677</i>	This Study
pAK0093	Suicide plasmid used to delete <i>fjoh1985</i> -1986	This Study
pAK0096	Suicide plasmid used to delete <i>fjoh3950</i> -3952	This Study
pAK0097	Suicide plasmid used to delete <i>fjoh3971</i> -3973	This Study
pAK0098	Suicide plasmid used to delete <i>fjoh4538</i> -4540	This Study
pAK0101	Suicide plasmid used to insert a rigid linker between <i>remG</i> and the <i>halotag</i> at the C-terminus of the encoded protein	This Study
pAK0102	Suicide plasmid used to truncate <i>sprD</i> to the N-terminal 1-633 residues of the encoded protein	This Study
pAK0103	Suicide plasmid used to truncate <i>sprD</i> to the N-terminal 1-309 residues of the encoded protein	This Study

Plasmid.ID	Description	Origin
pAK0104	Replicative plasmid used to overexpress <i>halotag</i> from an <i>ompA</i> promoter	This Study
pAK0105	Suicide plasmid used to fuse a <i>halotag</i> sequence to the C-terminal end of the <i>gldB</i> encoded protein	This Study
pAK0106	Suicide plasmid used to fuse a <i>halotag</i> sequence to the C-terminal end of <i>gldD</i> encoded protein	This Study
pAK0107	Suicide plasmid used to fuse a <i>halotag</i> sequence to the C-terminal end of the truncated <i>sprD633</i> encoded protein	This Study
pAK0108	Suicide plasmid used to fuse a <i>halotag</i> sequence to the C-terminal end of the truncated <i>sprD309</i> encoded protein	This Study
pAK0109	Suicide plasmid used to remove the 50 C-terminal residues from the <i>sprD</i> encoded protein in a <i>halotag::sprB</i> mutant background (e.g. sAK73)	This Study

7.3 List of primers

Table 7.3: Primers used in the creation of plasmids. Plasmids involved indicate the plasmids that used a given primer in its creation.

Primer ID	Plasmids involved	Primer sequence
priAK0035	pAK0016	TGACCCCGAAGCAGGGTTATa tctccaagtgaagctaattctttggca
priAK0036	pAK0016	CCGGTGAGCTctttgtggttatgggtgtg gattacaattgatccaagcaataac
priAK0037	pAK0016, pAK0017, pAK0021, pAK0109	actcgcaaaatttaatttctcatatgagcgcttg gagccac
priAK0038	pAK0016	tcaattgtaatcaccaccataaccacaaagAG CTCACCGG
priAK0039	pAK0016, pAK0017, pAK0021	tggctccaagcgctcatatgagaaattaaatttt gcgagtaggaggca
priAK0040	pAK0016, pAK0017, pAK0021	TTACGCCAAGCTTGCATGcct cttcaggcaaaacaagcgact
priAK0043	pAK0017	TGACCCCGAAGCAGGGTTATa aaaaaacaaaaatttaggaataaagaaaaaa atcgatcttct

Primer ID	Plasmids involved	Primer sequence
priAK0044	pAK0017	CCGGTGAGCTctttgtggttggtgcag gtacagaaattagaccg
priAK0045	pAK0017	ctaatttctgtacctgcaccaaccacaaagAG CTCACCGG
priAK0059	pAK0021	TGACCCCGAAGCAGGGTTATa atatcatctaagtcaagtcatttccaaatcaa cagc
priAK0060	pAK0021	AGATTTCGGTGAGCTctttttta attcaaagtttgataaaaacttgaaggaaaata tctttctaaaagc
priAK0061	pAK0021, pAK0109	ttatcaaactttgaattaaaaagAGCTCA CCGGAAATCTCCA
priAK0071	pAK0040, pAK0041	TATGCAGCGGAAAAATTTCGGt ttaagagcttcagcatcagcttttagctttattg
priAK0076	pAK0040, pAK0041	ATTACGCCAAGCTTGCATGct tagtgcatttttaatcatgacaactgcagc
priAK0080	pAK0042	GCAGCGGAAAAATTTCGGGgccca aagcggcagcaga

Primer ID	Plasmids involved	Primer sequence
priAK0083	pAK0042	CGCCAAGCTTGCATGCccaccgct ggtatgatctctcatat
priAK0144	pAK0040	tctaaaggctgcggaactatACCGGAAA TCTCCAGAGTAGacag
priAK0145	pAK0040	attcttatgccaggtgcgagcgcttgagcc acccgcagttcgaaaaagg
priAK0146	pAK0040	tgcggtggctccaagcgctgcgagcctgggca taagaatttgat
priAK0147	pAK0040	CTACTCTGGAGATTTCCGGTa tagttccgcagccttagatgg
priAK0148	pAK0041	tctaaaggctgcggaactatacccagcccaggc ttgcca
priAK0149	pAK0041	tgggcaagcctgggctgggtatagtccgcagc cttagatgg
priAK0150	pAK0041	atttcgcagctttgtccatgcagcctgggc
priAK0151	pAK0041	attcttatgccaggtgcgatggacaaagact gcgaaatgaag
priAK0161	pAK0048, pAK0076	ATTACGCCAAGCTTGCATGCa tgaaaaaattactcaaatgatctgcgttct

Primer ID	Plasmids involved	Primer sequence
priAK0162	pAK0048	ttaatcacggttattgatccagataaagtagttt tttgaatcatatagtagcttttcttgc
priAK0163	pAK0048	ttcaaaaaactactttatctggatcaataaccgt gattaaataatgaagaaaattttactattcata ac
priAK0164	pAK0048	TATGCAGCGGAAAAATTCGGg gccttagctttttctgctgcagctaaac
priAK0172	pAK0050	ATTACGCCAAGCTTGCATGCa gaagcagcagcaactgcag
priAK0173	pAK0050	ttattgcatttgcagcaatggtaacaggaggcg gaagaaaataataataacttagt
priAK0174	pAK0050	tttcttcgcctcctgttaccattgctgcaaatgc aataaacttcttcatataaatt
priAK0176	pAK0050, pAK0051	ATGCAGCGGAAAAATTCGGG agcagctaaaaaaactacaaccgcta
priAK0177	pAK0051	acttagtaattccacatatccattgctgcaaatg caataaacttcttcatataaatt

Primer ID	Plasmids involved	Primer sequence
priAK0178	pAK0051, pAK0053, pAK0054	ATTACGCCAAGCTTGCATGct taacaggaagttgtcctaaatgtcaaaaagaga gcatgta
priAK0179	pAK0051	ttattgcatttgcagcaatggatatgtggaatta ctaagtgccttaaatccaaattatacaaaaaact
priAK0185	pAK0053	acttagtaattccacatatcatttcttactttcat cgtggtatcattataagatattgaac
priAK0186	pAK0053	ccacgatgaaagtaagaaatgatatgtggaatt actaagtgccttaaatccaaattatacaaaaaac
priAK0187	pAK0053	ATGCAGCGGAAAAATTCTGGGt gcttttggtttgatgaatgaaaaattcgaagctg caaatacat
priAK0190	pAK0055	ATTACGCCAAGCTTGCATGct gttgatttagaaaaagcaggaatcaaaattatt caaatcg
priAK0191	pAK0055	taagattagaagtttttagatagaatttttttca taatttgggtatattttatttttagcattcct

Primer ID	Plasmids involved	Primer sequence
priAK0192	pAK0055	aaattatgaaaaaattctatctaaaaacttct aatcttaaattatatattcaatttcagaaagcac ct
priAK0193	pAK0055	ATGCAGCGGAAAAATTCGGG gaataggagtatactgaaaatgccagcataatt gaggttaaaatattct
priAK0196	pAK0056	ATTACGCCAAGCTTGCATGCa ctattggctgatctccccagtatgtcgg
priAK0197	pAK0056	ttattttattcgtacttagctttaattttcaacat aattatttggggattagaaaaataataacgcaa t
priAK0198	pAK0056	taattatgttgaaaattaaagctaagtacgaat aaaataaacaatcaaatacctctgtaaaaaga gca
priAK0200	pAK0056	ATGCAGCGGAAAAATTCGGGt atgaaaggtgatactttaattgctgattatactt ttaattctgaagggg

Primer ID	Plasmids involved	Primer sequence
priAK0201	pAK0057	ATTACGCCAAGCTTGCATGCc aaaattgggttacggatattatttaagtaacgag ga
priAK0203	pAK0057	tagttttaaaataaatccttatatagttttctcat tgtatctatttttttgtacaaatatgctatcgg
priAK0204	pAK0057	atacaatgagaaaactatataaggattttttt aaaactaaataataatgaaacatacactaatta tagg
priAK0206	pAK0057	ATGCAGCGGAAAAATTTCGGGt ggttcaacatcatctggaagtgtaacatttttaa gtaaattatttacc
priAK0207	pAK0058	tttaattatttttttctacgctttccatttcatt ttgtaacacaaaacaacaacataac
priAK0208	pAK0058	acaaaatgaaatgggaaagcgtagcaaaaaaa taattaaagaaactacgttttccaaatcttt
priAK0209	pAK0058, pAK0069	ATTACGCCAAGCTTGCATGCt ttacagtcacagttttcagtaattactgaggct
priAK0212	pAK0058, pAK0069	ATGCAGCGGAAAAATTTCGGG gaaggaacaactccaggcatatctacagc

Primer ID	Plasmids involved	Primer sequence
priAK0213	pAK0054	tcatgatgaaagtaaggaatgatatgtggaatt actaagtgccttaaattcc
priAK0214	pAK0054	acttagtaattccacatatcattccttactttcat catgataaatattataagaaattaaacgtaatt t
priAK0216	pAK0054	ATGCAGCGGAAAAATTCGGGc tactattggtgctaaattacttgattttgattttg aagttggttta
priAK0239	pAK0064, pAK0068, pAK0076	ATTACGCCAAGCTTGCATGCc tgcaatttggactggttggtgtaaaattataatt tg
priAK0240	pAK0064, pAK0068	tattaattctcaacttttactatgaatagtaaaa ttttcttcattatttaatacacggttattgatccttt
priAK0241	pAK0064, pAK0068	agaaaattttactattcatagtaaaagttgaga attaataaatataaaaatataaagaggcttctgg aaac
priAK0242	pAK0064, pAK0068	TTATGCAGCGGAAAAATTCGt tagcaagaaaacgtactatatgattcaaaaaac tactttatcttt

Primer ID	Plasmids involved	Primer sequence
priAK0251	pAK0069	CTACTCTGGAGATTTCCGGTt aattaaagaaactacgttttccaaatcttg
priAK0252	pAK0069	caaagatttgaaaacgtagtttctttaattaA CCGGAAATCTCCAGAGTAG
priAK0253	pAK0069	acaagtatttgtttctgtagcaaaaaaGAA ATCGGTACTGGCTTTCc
priAK0254	pAK0069	gGAAAGCCAGTACCGATTTCTt tttttgctacagaaacaaaatacttgt
priAK0270	pAK0076	caaaaaactactttatctgggtaaaagttgaga attaataaatataaaatataaagaggcttctgg a
priAK0300	pAK0087	tttatattttatatttattaACCGGAAAT CTCCAGAGTAGacag
priAK0301	pAK0087	caatcgtaaaagttgagaatGAAATCGG TACTGGCTTTTCcattcga
priAK0308	pAK0082	atccatgggctatcactagtgagaagcccttc aggcaaaaca
priAK0311	pAK0082	cccgggtcgacgagctcttattaattctcaactt ttacgattgccattttccc

Primer ID	Plasmids involved	Primer sequence
priAK0326	pAK0076	tattaattctcaacttttaccagataaagtagt tttttgaatcatatagtagcttttcttgctaagtc tatacgctcaa
priAK0349	pAK0088	cccgggtcgacgagctcttattatTTTTTgctac agaaacaaaataacttgtttccgtcaccc
priAK0350	pAK0088	gtaacaatccatgggctatcgcgcaaactacta atgccc
priAK0359	pAK0091	GGGTTATGCAGCGGAAAAAT gacttttacacttcactcttttgggtagaaatcc
priAK0361	pAK0091	tgtctatgcgtaactctactgtaagtttgaattt gattaaatgaaaaaggctttctaggaat
priAK0362	pAK0091	taatcaaattacaaacttacagtagagttacgc atagacagactcg
priAK0364	pAK0091	GATTACGCCAAGCTTGCATGc gccgatgttttatcgcc
priAK0365	pAK0092	GGGTTATGCAGCGGAAAAAT agtaaaatatcagcaagaaatttggtcaatgtt gg

Primer ID	Plasmids involved	Primer sequence
priAK0367	pAK0092	agctaaccattatgaaattagcagttaattaaa aaaatattatggttataaaatctgtaaacggaa aagc
priAK0368	pAK0092	atattttttaattaactgctaatttcataatggt tagctagtttatataatttttgctctcttattaa
priAK0370	pAK0092	GATTACGCCAAGCTTGCATGa aggtccatttcctatcaaaacaccaagtca
priAK0372	pAK0093	GATTACGCCAAGCTTGCATGa tacttatatttcggttgaaaaaatataacgaagc aaaattgatattttct
priAK0373	pAK0093	acctcagtatgataaaatttaaagggttttagtt aatcaactaaaccattttcgact
priAK0374	pAK0093	ttgattaactaaaaccctttaaattttatcatact gaggtattataatttgcaaatactatttaaagtg c
priAK0375	pAK0093	GGGTTATGCAGCGGAAAAATc tgcgggcgtagaaatttgagtgga

Primer ID	Plasmids involved	Primer sequence
priAK0390	pAK0096	GGGTTATGCAGCGGAAAAAT ggaataacaacaaaacttacaaatTTTTTactg aaatTTTccttg
priAK0391	pAK0096	aatgaactTTgttatatagatTTTatcataaata actcgattatTTTattacctgatgaagattatag g
priAK0392	pAK0096	tcgagttatTTatgataaaatctatataacaaag ttcattTTTTaaatTTtagTTTtaattaaaaacc g
priAK0394	pAK0096	GATTACGCCAAGCTTGCATGg aatattacctTTTaaactTTTtcgattTTTgaatat aaatTTcctTTgaa
priAK0395	pAK0097	GGGTTATGCAGCGGAAAAAT ggattaattaatattaaactgaaaacggcaaaa gcaaattcatgga
priAK0397	pAK0097	tcccgaagcctatggaacaaaaaatgtaacca aaaatcgaccttaaacatTTTaat
priAK0398	pAK0097	cgatTTTtgggttacatTTTtgttccataggctt cgggattgaaaagtgga

Primer ID	Plasmids involved	Primer sequence
priAK0400	pAK0097	GATTACGCCAAGCTTGCATGg aggaaagaaagaacagtttttaaattaatgaa agatgaaatttaatt
priAK0402	pAK0098	GGGTTATGCAGCGGAAAAAT gatggtaccaattcaggacttaatttttg
priAK0403	pAK0098	taaaatgatttaaaatggaagcattgtaactta gaaaaactgactaaaaaaaaataaaagctgtct at
priAK0404	pAK0098	gtttttctaagttacaatgcttccattttaaatca ttttaagggtatacttgatatacttgtttaatca
priAK0406	pAK0098	GATTACGCCAAGCTTGCATGg aagctgttcagagttatgatattcacgttgctta tta
priAK0420	pAK0103	ATTACGCCAAGCTTGCATGCc ctcaaacagcaatagattttaaaatgtaaate cagt
priAK0421	pAK0103	TATGCAGCGGAAAAATTTCGGa tcataagtaggtccctcatatgtaatacgag

Primer ID	Plasmids involved	Primer sequence
priAK0423	pAK0103	tttatatatttatatttattatgctttggcttttgca tcagcaact
priAK0424	pAK0103	ctgatgcaaaagccaaagcataataaatataa aatataaagaggcttctggaaaccagaag
priAK0452	pAK0104	gtaacaatccatgggctatcGAAATCGG TACTGGCTTTCcattcga
priAK0453	pAK0104	cccgggtcgacgagctcttaACCGGAAA TCTCCAGAGTAGacagc
priAK0471	pAK0105	gGAAAGCCAGTACCGATTTTCtt tcttaggtttatattttgacttttcgaaaatttct tttgcatctact
priAK0472	pAK0105	CTACTCTGGAGATTTCCGGTt aatgtcaaatacaataaaatcagaaattaaatt caacgtcgaattagac
priAK0474	pAK0105	TTATGCAGCGGAAAAATTCGg ccttattaccgcttcgggttctaaatacctt
priAK0475	pAK0105	caaaatataaacctaagaaaGAAATCG GTACTGGCTTTCcattcg

Primer ID	Plasmids involved	Primer sequence
priAK0476	pAK0105	TTACGCCAAGCTTGCATGCtga taccaccgaaattaaataatttctaaagtcttag ctttattaactaa
priAK0477	pAK0105	ttttattgtatttgacattaACCGGAAAT CTCCAGAGTAGacagc
priAK0479	pAK0106	TACGCCAAGCTTGCATGCtacg ttacagacagtgcagttgttcagttataaaa
priAK0480	pAK0106	TGCAGCGGAAAAATTCGGGtt ctttcgggaagttgtgacaacttacaattcaa t
priAK0482	pAK0106	gGAAAGCCAGTACCGATTTTCtt tccactttaagtttccattatacgtgcatgtc atTTTTgatataa
priAK0483	pAK0106	tggaaactttaagtggaagGAAATCGG TACTGGCTTTTCcattcg
priAK0484	pAK0106	CTACTCTGGAGATTTCCGGTt aaaaaataattaagaaataaaaaacggcggtc aattgaacgccgtt

Primer ID	Plasmids involved	Primer sequence
priAK0485	pAK0106	tatttcttaattattttttaACCGGAAAT CTCCAGAGTAGacagcc
priAK0487	pAK0107, pAK0108	tttatattttatatttattaACCGGAAAT CTCCAGAGTAGacagc
priAK0488	pAK0107	ctgatgcaaaagccaagcaGAAATCG GTACTGGCTTTTCcatt
priAK0493	pAK0108	acagaaattactattacggaGAAATCGG TACTGGCTTTTCcat

7.4 CXMS R-code

```
library(limma)
library(stringr)
library(tidyverse)
library(ggrepel)

# A gist that allows you to add new aes scales
# devtools::source_gist("https://gist.github.com/eliocamp/eabafab2825779b88905954d84c82b32")

# Set the dir to the one containing the data files
setwd('D:/Dropbox/Oxford/Ben_Berks/Mass_Spectrometry')

#####
# PREPARE THE DATA FOR
#####

# Load the data
df_cxms_3 = read.csv('CXMS_GldJ_3/AndreaKjaer_MQ\\output_submission\\181129.csv')
df_cxms_1 = read.csv('CXMS_GldJ_1/AndreaKjaer_MQoutput_181120.csv')
df_psort = read.csv('psortb--results.txt', sep = "\t") # For subcellular localizations
```

```

df_uniprot_id_lookup = read.csv('uniprot_gi_ids.csv', sep = "\t") # For translating gene ID to protein ID

df_t9ss_data = read.csv('t9ss_proteins.csv') # Detailed info about t9ss proteins

##### Preparing the psort dataframe (with my own annotations) for annotating plot #####

#Renaming uniprot gi column (long name from uniprot) to be gi
colnames(df_uniprot_id_lookup)[2] <- "gi"

#Splitting the psort seqID column to make a single column that only contains the gi number
split_seqid <- data.frame(do.call('rbind', strsplit(as.character(df_psort$SeqID),'|',fixed=TRUE)))

df_psort$gi <- split_seqid$X2

#Doing merge to get my psort data with uniprot IDs
df_psort <- merge(df_psort, df_uniprot_id_lookup, by = "gi")

#Gonna adjust my psort localization data with my manually constructed t9ss data
# I make sure the colnames are the same for the "common" column between the two
colnames(df_psort)[41] <- "Protein.IDs"

#Left join makes two column ("column".x and "column".y). When they are not
#The same value, "column".y gets a nan.
df_psort <- dplyr::left_join(df_psort, df_t9ss_data, by = "Protein.IDs", copy = TRUE)

#I am using the fact that there are na's in the "column".y to index
#"column".x and replace the "column".x whenever there is a value in "column".y
df_psort$Final_Localization.x[!is.na(df_psort$Final_Localization.y)] <- df_psort$Final_Localization.y[!is.na(df_psort$Final_Localization.y)]
##### END Preparing the psort dataframe (with my own annotations) for annotating plot #####

##### Merging and preparing df_all by merging the cxms-psort first and then merging cxms-cxms #####

#Merging the df_cxms dataframes with my annotation psort dataframe
df_cxms_3 <- merge(df_psort, df_cxms_3, by = "Protein.IDs")
df_cxms_1 <- merge(df_psort, df_cxms_1, by = "Protein.IDs")

#I want to find the average of every proteins ctrl and xlink intensity in all three datasets
df_all <- merge(df_cxms_3, df_cxms_1, by = "Protein.IDs", all=T)

#The merging creates NAs in places where I do not have the proteins in both cxms dataframes.
# To avoid problems downstream, I set their values to 0.
df_all[c("Intensity.2d", "Intensity.45D", "Intensity.X.Link", "Intensity.2x", "Intensity.45x", "Intensity.CTRL")] [is.na(df_all[c("Intensity.2d", "Intensity.45D", "Intensity.X.Link", "Intensity.2x", "Intensity.45x", "Intensity.CTRL")])] = 0,]

#Alternatively try to omit them:
df_all <- df_all[rowMeans(is.na(df_all[c("Intensity.2d", "Intensity.45D", "Intensity.X.Link", "Intensity.2x", "Intensity.45x", "Intensity.CTRL")])) == 0,]

# Creating some nicer names for plot annotation by splitting the fasta headers to get the GN= name (gene name)

```

```

df_all$nice_names <- stringr::str_match(as.character(df_all$Fasta.headers.x), "(GN=)([a-z|0-9]*)")[,3]
##### END Merging and preparing df_all by merging the cxms-psort first and then merging cxms-cxms #####

##### Setting up data for Volcano Plot #####

#Define the groups of data

grp1 = c("Intensity.2d", "Intensity.45D", "Intensity.X.Link")
grp2 = c("Intensity.2x", "Intensity.45x", "Intensity.CTRL")
grp_all = c(grp1, grp2)

#Set min value and max value to handle inf errors (doing it )
for (column in grp_all){
  df_all[column][df_all[column] == 0] <- min(df_all[column][df_all[column] > 0])
}

df_all[grp_all] <- log2(df_all[grp_all])
#boxplot(df_all[grp_all])

df_all[grp_all] <- normalizeBetweenArrays(df_all[grp_all])
#boxplot(df_all[grp_all])

#Calculate the pvalue for each protein
ttestFun <- function(df, grp1, grp2) {
  x = df[grp1]
  y = df[grp2]
  x = as.numeric(x)
  y = as.numeric(y)
  results = t.test(x, y)
  results$p.value
}

df_all$rawpvalue = apply(df_all, 1, ttestFun, grp1 = grp1, grp2 = grp2)

#Calculate the mean intensity of all proteins
control = apply(df_all[,grp1], 1, mean)
test = apply(df_all[,grp2], 1, mean)

#Use the mean to calculate fold change ratios
df_all$foldchange = test - control

#Points of interests
df_sp <- rbind(df_all[df_all$Protein.IDs %in% df_t9ss_data$Protein.IDs,],df_all[df_all$foldchange > 4 & df_all$rawpvalue < 0.05,])

df_sp <- df_sp[!duplicated(df_sp),]

##### END Setting up data for Volcano Plot #####

```

```
##### Doing the actual plotting #####

colours = c("Cytoplasmic" = "black", "Outer_Membrane" = "red", "Cytoplasmic_Membrane" = "purple", "Unknown" = "grey", "Periplasmic" = "blue", "Extracellular" = "green")

volcano = ggplot(data = df_all, aes(x = foldchange, y = -1*log10(rawpvalue)))
volcano = volcano + geom_point(aes(color = df_all$Final_Localization.x.x))

#Annotate the points of interest on the plot and color the font so it matches the subcel localization
volcano <- volcano + geom_text_repel(data = df_sp,
                                     aes(x = foldchange,
                                          y = -1*log10(rawpvalue),
                                          color = Final_Localization.x.x,
                                          label=nice_names),
                                     size = 2)

volcano + theme_classic(base_size = 2)

# Coloring subcellular localization by defined colors
volcano <- volcano + scale_color_manual(values = colours)

volcano
```

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