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Mutations close to the peptidoglycan-binding region of the stators of the bacterial flagellar motor influence phenamil resistance

ii. Running Title:
Mutants in PG-binding motif of MotB influence phenamil resistance

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Summary:

The bacterial flagellar motor (BFM) is a molecular complex which powers the rotation of the filament that propels swimming bacteria. Rotational torque is generated by harnessing the flow of ions through ion channels known as stators which couple the energy from the ion gradient across the inner membrane to rotation of the rotor. Here we use error-prone PCR to introduce single point mutations into the sodium-powered Vibrio/E. Coli chimeric stator PotB. We then select for motors that exhibit resistance to the sodium-channel inhibitor phenamil. We found that single mutations that inferred resistance to phenamil occurred at two sites: 1) the transmembrane domain of PotB, corresponding to the TM region of the PomB stator from *V. alginolyticus*, and 2) near the peptidoglycan (PG) binding region that corresponds to the N-terminal region of the MotB stator from *E. coli*. We corroborated our swim plate observations with single cell rotation assays to confirm that individual cells could drive rotation of flagellar motors in the presence of up to 100 µM phenamil. Our results demonstrate that it is not only the pore region of the stator that moderates the effect of motility in the presence of ion-channel blockers. We hypothesise that stator dwell time on phenamil on the sodium ion flow, but that the changes far from the pore can also affect the motor, affected by mutations in the PG region, can allow motors to function in the presence of phenamil by allowing multiple semi-functioning stators to persist on the motor and drive flagellar rotation.
Introduction

Motility imparts a large benefit to organisms competing for sparse nutrients. The bacterial flagellum is the oldest known form of motility (Rossmann and Beeby 2018), consisting of propeller-like filament that rotates under the power of the bacterial flagellar motor (BFM). The BFM is 40 nm in diameter, powered by ion transit across the cell membrane through the stator protein complex, a heterodimer which forms a selective ion channel that transduces chemical energy into mechanical torque (Minamino et al. 2018). While most stators are proton powered (Sowa and Berry 2008), some from marine habitats are sodium powered (Yorimitsu and Homma 2001), others are powered by both sodium ions and protons (Paulick et al. 2015) and recently some have been discovered that are even powered by large divalent cations (Imazawa et al. 2016). In general, flagellar motors have adapted to function in various environments where bacteria live and survive (Terashima et al. 2017; Rossmann and Beeby 2018; Chaban, Coleman, and Beeby 2018). BFM diversity creates an ideal case study to investigate how macromolecular complexes adapt to different environments.

The majority of motors are powered by either protons or sodium. In E. coli the energy from the proton-gradient is harnessed by the transit of protons through a heterodimeric stator complex is composed of MotA4MotB2 (Minamino et al. 2018). In Vibrio strains this heterodimer is the sodium-powered PomA4PomB2. The total complex, in both cases, consists of four transmembrane (TM) domains of the A-subunit, a single TM domain of the B-subunit, and a large periplasmic region of the B-subunit which consists of a plug segment and a peptidoglycan-binding (PG-binding) region (Seiji Kojima and Blair 2001; Seiji Kojima et al. 2009). Recently, the structural rearrangements in PG-binding and stator activation were resolved, indicating that conformational rearrangements of the linker between the PG-binding region and the TM-domain are critical in stator activation (Seiji Kojima et al. 2018).

Whilst crystal structures have been solved for the C-terminal domains of MotB in multiple species (Roujeinikova 2008; Seiji Kojima et al. 2009; Zhu et al. 2014), the full structure of the TM-domain in any species has yet to be resolved beyond low-resolution cryoEM of the entire stator complex (Yonekura, Maki-Yonekura, and Homma 2011). Much of our understanding of stator function in the TM-domain relies on mutagenesis (Sharp, Zhou, and Blair 1995; Yakushi et al. 2006). Chimeric B-subunits have been engineered that combine the N-terminal peptidoglycan binding motif of the MotB subunit with the transmembrane domain of PomB that forms the ion-channel (in complex with two subunits of MotA) (Asai et al. 2003) (Fig. 1). These hybrid PomA4PotB2 stators utilise sodium motive force to drive flagellar rotation and can bind to the peptidoglycan (PG) layer thus enabling E. coli to swim under sodium-motive force. They have enabled the function of the motor to be investigated at low sodium concentration, and thus low energisation (Sowa et al. 2005; Lo et al. 2013).

Since this chimeric stator complex is driven by sodium ion flow across the inner membrane, it is natural to examine how sodium channel blockers affect this process. Phenamil, a known sodium-channel blocker (Garvin et al. 1985), has been used to probe sodium-interaction sites in sodium driven motors in situ in Vibrio species (Sugiyama, Cragoe, and Imae 1988; T. Atsumi et al. 1990). Work in V. alginolyticus demonstrated that phenamil resistance could be induced by mutations at the cytoplasmic end of the TM-domain of the stator complex (S. Kojima et al. 1997, 1999). V. parahaemolyticus has been widely studied as a model for dual-powered
motility; it has lateral flagella that are proton powered but polar flagellar that are sodium powered (Tatsuo Atsumi, McCartert, and Imae 1992). Directed evolution approaches have been applied to *V. parahaemolyticus* to examine which spontaneous mutations might induce resistance to phenamil (Jaques, Kim, and McCarter 1999). Jaques et al. selected for resistance to phenamil by selecting flagellar motors that were motile in the presence of 40 µM phenamil methanesulfonate, yet interestingly only observed a single mutant in the TM-domain that resulted in phenamil resistance.

Here we engineered a plasmid construct with enzyme cut sites adjacent to PotB to examine the effects of randomly generated mutations on motility. We screened using phenamil to measure the frequency and location of mutations that imparted phenamil resistance in the sodium powered PomA<sub>4</sub>PotB<sub>2</sub> chimera. We induced mutations at a controllable rate using error-prone PCR (EP-PCR) and screened large populations of cells using streaking of transformed EP-PCR product onto swim agar in streaks for screening and subsequent sequencing. This allowed a high-throughput screen to determine which mutations have resulted in strains that were functional in the presence of phenamil. By examining the structural and sequence location of these mutations, we have assembled a model that outlines the relationship between PG-binding and stator activity.

![Figure 1: Schematic and structure of the stators of the bacterial flagellar motor.](image)

(A) The stator complex in *E. coli* consists of a heterodimeric complex composed of MotA<sub>4</sub>MotB<sub>2</sub>. Each stator has two ion channels that are composed of 4 TM domains of MotA and 1 TM domain of MotB. (B) The PotB chimera consists of residues 1-50 from *V. alginolyticus* PomA and residues 59-308 of *E. coli* MotB with TM, plug, PGB and ompA-like regions indicated on the schematic respectively. Structure in A from (Nishino et al. 2015).

**Results**

**Mutagenesis of PotB.**

We generated mutations across the entirety of the PotB protein using error-prone PCR (EP-PCR) as per Methods. We defined the following regions across the PotB protein (numbering via PotB residue number): the TM domain (20-42), the plug (44-57), the middle domain (57-140) and the peptidoglycan binding domain (188-214). We screened the combinatoric pool of mutagenesis outputs using swim streaking and characterised the motility of 30 EP-PCR plasmids that exhibited some motility when selected against phenamil resistance (Table S1). The distribution of all mutations in all 30 plasmids, including silent mutations that did not change the coded residue, is shown in Fig. 2.
Binning the mutations in bins of 10 showed that the mutations occurred evenly throughout the protein. When we categorised the mutations across regions of the protein, we observed that most mutants were in the ompA-like region surrounding the PG binding region, with a spike of mutations in the TM region.

**Point mutations in TM domain can impart phenamil resistance.**

Out of the 27 strains (Table S1), six exhibited a strong motility phenotype when motility was characterised in the presence of 20 µM phenamil. Of particular interest, we focused on plasmid EP-PCR-41 which contained only 3 mutations: F22Y, L28Q, K100Q and demonstrated increased motility in the presence of phenamil. To address the relative contributions of each mutation to the observed phenotype we synthesised single point mutants of each of the three mutations. Both F22Y and L28Q alone imparted phenamil resistance to PotB (Fig. 3), or together (pSHU149). Mutation K100Q appeared to offer no phenamil resistance and as such was classified a redundant mutation introduced by EP-PCR. These results indicate that single point mutations, particularly in the pore region of the TM domain of PotB, can impart phenamil resistance.

**Point mutations near PG-binding domain can impart phenamil resistance.**

Plasmid EP-PCR-31 displayed a phenamil-resistant phenotype and also had only three mutations: Y163F, M164V and L255M. This was targeted for further investigation as these mutations were all far from the TM domain. We again characterised the contributions of each mutation to motility and observed that Y163F and M164V each separately impart phenamil resistance and that L255M was a redundant mutation induced by EP-PCR (Fig. 3). When both mutations were combined in a single plasmid (pSHU146), phenamil-resistance was increased.
Figure 3: Mutants in TM-domain and PG-binding region induce phenamil resistance. Motility of either TM-domain mutants (A/B) or PG-binding mutants (C/D) was tested via 0.25% agar swim plating using the empty vector (pBAD33) and pSHU1234 as a negative and positive control respectively. A/C In the absence of phenamil, swimming of mutants is comparable to the positive control. B/D In the presence of 20 µM phenamil, the mutants are motile whilst pSHU1234 is severely limited. A/C incubated at 30˚C for 10 hours, B/D incubated at 30˚C for 18 hours.

**PG-binding double mutants rotate at lower sodium concentration than wild-type PotB**

To establish whether these mutants were generating torque from sodium motive force, or had undergone a change in ion-specificity, we tested swimming in minimal sodium-free media (Fig. S2). None of the mutants were motile in sodium-free media, yet all plasmids restored swimming in the presence of sodium and in the presence and absence of phenamil (Fig S3). We then tested individual cells for rotation whilst lowering the concentration of sodium to determine whether the dependence on sodium-motive force (SMF) had changed in these mutants (Fig. 4). This allowed us to confirm that our observed swim plate phenotype was caused changes in flagellar rotation, and account for any compensatory changes in growth or chemotaxis that might confound swim plate measurements. We examined the capacity of pSHU1234 and our mutant plasmids to restore swimming in ‘sticky-filament’ strains. These sticky-filament mutants lack the surface of the protein FliC and thus undergo hydrophobic interactions with glass that enables filaments to stick to glass and the rotation of the body be assessed in parallel (Kuwajima 1988). In comparison with pSHU1234, the mutants in the PG-region (pSHU146: Y163F/M164V) where highly motile at low sodium, with cells rotating at 6 Hz in the presence of only 0.3 mM Na⁺. This is in contrast to pSHU1234 restoring motility to < 2 Hz at the equivalent sodium motive force. In an opposite trend, mutations in the TM region (pSHU149: F22Y/L28Q) did not demonstrate swimming below 1 mM Na⁺ and had a lower maximum speed and slower response to increased [Na⁺].

Figure 4: Y163F/M164V is motile at less than 0.3 mM [NaCl]. (A) The PomAPotB wild-type exhibits single cell rotation from 0.3 mM Na⁺, increasing exponentially to a plateau above 6 Hz at full energisation. (B) PotB Y163F/M164V displays rotation at 0.3 mM Na⁺, with plateau above 6 Hz at full energisation. (C) potB F22Y/L28Q displays no rotation below 1 mM Na⁺, with a slower increase to a lower maximum speed plateau above 5 Hz and under 6 Hz. Inset in ABC shows zoom around 0-5 mM [Na⁺].
**TM-domain double mutants are not affected by increasing concentrations of phenamil.**

The response of single-cell speed with varying phenamil concentration was measured to examine the energy profile and structural basis of phenamil resistance in our two phenamil-resistant plasmids. In wild-type PotB (pSHU1234) tethered cell rotation speed was reduced significantly when as little as 1 µM of phenamil was introduced, and cell speed plateaued at 5 µM of phenamil. The PG-binding double mutant (PomAPotB Y163F/M164V) demonstrated the same plateau effect in response to increasing phenamil but the concentration dependence of the decrease in rotational speed was much lower, that is, the decay constants for a monoexponential fit were 0.8 mM⁻¹ and 0.2 mM⁻¹ for wild-type and Y163F/M164V respectively. Similarly, the $I_{50}$, the concentrations of phenamil at which the speed was half the maximum, was 0.9 mM and 3.1 mM for wild-type and Y163F/M164V respectively. In contrast, the speed of rotation for the double TM-domain mutant (PomAPotB F22Y/L28Q) exhibited no dependence on phenamil concentration up to 50 µM, and only a 46% reduction on maximum speed at 100 µM phenamil. This implies that the mechanism of resistance in the TM-domain mutants destroys much of the efficacy of phenamil altogether, whereas the PG-binding region mutants act to stabilise rotation in the presence of phenamil, but this stabilisation can be overcome as phenamil concentration is increased.

*Figure 5*: TM mutants impart phenamil resistance up to 100 µM. Dose response of averaged measurements of single-cell rotation speed with varying concentration of phenamil for: (A) PomAPotB wild-type, (B) double TM-domain mutant (PomAPotB F22Y/L28Q) and (C) double PG-binding mutant (PomAPotB Y163F/M164V). Tethered cell data is fit with monoexponential decay curve to generate time constants for decay (PomAPotB wild-type: 0.8 mM⁻¹; PomAPotB Y163F/M164V: 0.2 mM⁻¹; PomAPotB F22Y/L28Q: 0.007 mM⁻¹).
F22Y is common and clusters in phylogenies along MotB/PomB lines; L28Q is very rare.

A large low-level phylogeny of 948 MotB homologues was assembled which clearly divided into clades (Fig. 7A). Examination of a curated subset of 32 commonly studied species (Fig. 7B) was examined to look at patterns at the sites of the mutations studied in this work. Parsimony trees were reconstructed at each node of the smaller phylogeny and the sequence identity at each node estimated to map trends in mutation across the phylogeny. At the F22Y site, residues clearly clustered according to MotB/PomB identity (Fig. 7B, left), which is putatively linked to proton/sodium motility. However, at the L28Q site (Fig 7B, right), the 32 curated species did not divide along MotB/PomB lines, in fact were in most cases leucine and in close relatives of E. coli were alanine. In fact, over the entire 948 species in the larger phylogeny, only one species had a glutamine at that site: Myxococcus fulvus.

Figure 6: Parsimony Reconstruction for Y22 and L28. (A) Overall phylogeny of 948 MotB homologs showing clear cladding. (B) Parsimony reconstruction for site Y22 (PotB numbering) shown in colour over subset phylogeny of 34 representative species as labelled. (C) Sequence alignment across transmembrane domain of MotB/PomB showing conserved D24, and Y22/L28 mutations tested in this work. (D) Parsimony reconstruction for site L28A (PotB numbering) shown in colour over subset phylogeny.
**Y163F/M164V are both rare and do not cluster along MotB/PomB lines.**

Similarly, we aligned the sequences in the section 156-214, including the PG-binding site, and performed parsimony reconstructions at each node of our curated phylogeny. In both Y163F and M164V the mutations did not cluster according to MotB/PomB lines. A phenylalanine at site 163 was very rare across the full phylogeny, occurring only in *Yersinia pestis biovar Orientalis*. The valine at site 163 was observed in 13 species, mostly among members of the *Spirochaetes* and *Oceanospirilla* families (Table S3).

![Figure 7: Parsimony Reconstruction for Y163 and M164.](image)

(A) Parsimony reconstruction for site Y163 (PotB numbering) shown in colour over subset phylogeny of 34 representative species from Fig. 5. (B) Sequence alignment over peptidoglycan binding site of MotB/PomB showing Y163/M164 mutations tested in this work. (C) Parsimony reconstruction for site M164 (PotB numbering) shown in colour over subset phylogeny.
Figure 8: Model of activity. Schematic showing effects of mutations with respect to phenamil and sodium flow through stator complex. The pool of stator complexes (PomA$_4$PotB$_2$) diffuse in the inner membrane, and when they bind to the motor the PG-binding region pulls with tension on the transmembrane domains of PomA and PotB [refKojima]. (left) Mutations in the 163/164 site affect PG-binding and the duration for which a stator is bound to the motor (dwell time). This results in a slower off rate with respect to wild-type stators and a larger steady-state population of stators bound to the motor. In the presence of phenamil each stator has reduced functionality but in sum, with many stators, the motor is functional. (right) Mutations at the TM-domain near the putative phenamil binding site affect the binding and release of phenamil, thus phenamil is bound more weakly, and turns over more rapidly, allowing sodium ions to pass through the channel to generate motor rotation.

Discussion

We have examined the effect of mutations in both the transmembrane domain and the peptidoglycan binding region of PotB. We observed that single point mutations in the TM region at L28 and F22 were sufficient to impart phenamil resistance. The mutation at F22Y is highly clustered around Pom/Mot stator subunits in varying species and presumably linked to sodium/proton specificity, which may that role residue F22 not only plays a role in sodium binding but also phenamil-binding and sodium blocking. We observed specific mutations at Together with a mutation we observed as an EP-PCR product at L28Q, these two mutations enabled phenamil resistance across a large range of phenamil concentrations, with stators remaining functional at concentrations of phenamil as high as 100 µM phenamil. This high tolerance to phenamil implies that they have affected or removed the binding site of phenamil inside the pore region of the stator complex.

Similarly, in the PG-binding region we observed that two clustered single mutations were also sufficient to impart phenamil resistance. The PG-binding region is used to bind the stators in place so that they act on the motor. With these two mutations at Y163/M164 we observed a decaying concentration dependence of rotation to phenamil. These sites are far from the pore, which is expected to be the phenamil binding site, thus we propose that the mechanism of resistance was not related to altering the efficacy of binding of phenamil. We propose that resistance was induced via changes to the binding strength of PotB to the PG layer in *E. coli*. This in turn would be sufficient to alter the dwell time of the stator on the rotor, that is, would result in more stators being bound more often (Fig. 8). In turn, if each stator is fractionally functional, or occasionally able to pass a sodium ion in the presence of phenamil, then in total, with more stators on the rotor, these sodium-powered flagellar motors are able to function...
in the presence of phenamil. This model needs to be further tested using single cell studies and combined single molecule fluorescence to quantify exactly and changes in the dwell time of mutant stators on the motor, the on and off rates of the stators binding to the motor, and how these properties change as a function of external sodium concentration.

Phenamil resistance has been studied in other species, most notably in *Vibrio parahaemolyticus*. This system is of interest as it has two motors, one sodium powered and one proton powered and phenamil has been studied previously as a drug target for this pathogen(Tatsuo Atsumi, McCartert, and Imae 1992; Jaques, Kim, and McCarter 1999). Jaques et al. used natural selection over 12-18 days to screen for phenamil resistance(Jaques, Kim, and McCarter 1999). They observed a single mutation in the TM-domain, but did not observe the mutations that we saw in this paper. Their experiment, occurring through natural mutagenesis and evolution in a laboratory setting will respond to pleiotropy and epistasis and also inherent directionality in the process of stator adaptation. This means that the order of specific mutations can restrict the possible outcomes. Naturally, our mutation at L28Q was indeed very rare across our set of 948 MotB homologues, occurring only once in *Myxococcus fulvus* ~0.1% of the species we examined. EP-PCR however, is agnostic to the order of mutations and will insert mutations evenly across the protein of interest. This highlights the power of EP-PCR to explore novel mutations and to compare historical evolutionary occurrences with those that can be discovered via directed and laboratory-assisted experimental evolution.

Our experimental system allows us to examine phenotypes to determine which amino acids have a functional cause in changing a phenotype. This allows us to address a common puzzle in phylogenetics: because both mutations and traits are inherited on the phylogeny, they are non-independent, and can thus exhibit “accidental” correlation without a functional relationship(Uyeda, Zenil-Ferguson, and Pennell 2018). In the case of MotB, every clade has some amino acids unique to it, but this will be true whether or not those residues have a functional role in the clade’s motility phenotype. For example, we can characterise phenamil resistance and adaptation in ion-source to measure whether changes on the phylogeny are associated with a particular ion source, or a particular habitat, across a number of independent transitions. This allows us to quantify which amino acids, potentially in which order, have been part of historic adaptation events such as sodium to proton based bacterial motility. In turn, we can use these approaches to tease out the causality of events that constrain natural adaptation.
Experimental Procedures

Bacterial strains and plasmids
Bacterial strains and plasmids used in this study are listed in SI. For swim plating, plasmids were transformed into RP6665 (∆motAmotB) (Block, Blair, and Berg 1989) to restore motility. For tethered cell assays, plasmids were transformed into JHC36 (∆cheY fliC-sticky ∆pilA ∆motAmotB) (Kuwajima 1988; Inoue et al. 2008).

Error-prone PCR
Plasmid pSHU1234 was constructed in a pBAD33 backbone with unique cut-sites for NdeI and PstI directly upstream and downstream of PotB. Error-prone PCR was executed on pSHU1234 using primer 1176 and primer 0104 (Table S2), taq polymerase, unbalanced dNTPs and Mn²⁺, as per (Wilson and Keefe 2000). The post-PCR product and pSHU1234 was then digested and ligated with NdeI and PstI and transformed into RP6665(∆motAB).

Mutant isolation
Transformant was streaked in lines on low agarose motility plate (Fig. S1) consisting of 0.25% TB soft agar, 25 µg/mL chloramphenicol, 1 mM arabinose and 20 µM phenamil. These plates were incubated at 30°C for 20 hours. Flares were selected from these plates and single colonies were generated. These colonies were harvested, the plasmid was purified, transformed again into RP6665 and motility was tested as below. Specific mutants derived from EP-PCR outcomes were engineered using a standard site-directed mutagenesis protocol with forward and reverse primers (Supplementary Materials), template, and high-fidelity DNA polymerase (Pfu Ultra).

Motility assays
Motility testing was carried out using motility plate assays. Phenamil to a final concentration of either 0, 20 or 100 µM was added to the plates. Swim plates were inoculated from single colonies by toothpick. Plates were incubated for 10 or 18 hours respectively, as noted.

Sequence analysis
Sequencing was executed commercially by FASMAC (Japan) using forward and reverse primers and template (Table S2). Three primers were used to read the entire PomAPotB region.

Single cell speed assays
Cells were adhered spontaneously to glass coverslips via a sticky-filament and then imaged onto a CMOS camera at 60 frames per second thorough 40x objective. Rotation of tethered sticky-filament cells was analysed using custom software based upon LabView (National Instruments).

Phylogenetics and sequence diversity of MotB homologs
To survey the known flagellar diversity for amino acid substitutions similar to the upmotile mutations discovered by experiment, we estimated a very large phylogeny of MotB and related proteins. As our goal was exploratory, we chose faster heuristic methods feasible for a large dataset, rather than Maximum Likelihood or Bayesian methods that would be
impracticably slow. 948 MotB homologs were assembled by searching UniProt90 against key study taxa. They were aligned using Clustal Omega (Larkin et al. 2007) on its most thorough settings (5 iterations of re-alignment). The phylogeny was then estimated with Quicktree, a neighbour-joining method suitable for large datasets (Howe, Bateman, and Durbin 2002). The phylogeny was midpoint-rooted in FigTree (Rambaut [2015] 2018). As these are fast heuristic methods based on a single protein, deeper branches and rooting should be considered uncertain, and we make no claim about e.g. inter-phylum relationships, a very difficult phylogenetic problem (Pallen and Matzke 2006; Abby and Rocha 2012; Shih and Matzke 2013; Koonin 2016). However, the tree was adequate for the purpose of showing relationships between close relatives, and for surveying MotB sequence conservation and diversity.

To further explore conservation and diversity at key positions of the alignment, ancestral amino acids were reconstructed on the phylogeny using parsimony in Mesquite (Maddison and Maddison 2015). The 948-protein tree was subset to key study taxa for Fig. 6 and Fig. 7.

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Author Contributions
TI collected and analysed data. RI collected and analysed data. NJM analysed data and wrote the manuscript. YS conceived and supervised the project, collected and analysed data, and wrote the manuscript. MABB conceived and supervised the project, analysed data, and wrote the manuscript.

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