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RESEARCH ARTICLE

Weak Interactions between Salmonella enterica FlhB and Other Flagellar Export Apparatus Proteins Govern Type III Secretion Dynamics

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Abstract

The bacterial flagellum contains its own type III secretion apparatus that coordinates protein export with assembly at the distal end. While many interactions among export apparatus proteins have been reported, few have been examined with respect to the differential affinities and dynamic relationships that must govern the mechanism of export. FlhB, an integral membrane protein, plays critical roles in both export and the substrate specificity switching that occurs upon hook completion. Reported herein is the quantitative characterization of interactions between the cytoplasmic domain of FlhB (FlhBC) and other export apparatus proteins including FliK, FlhAC and FliI. FliK and FlhAC bound with micromolar affinity. KD for FliI binding in the absence of ATP was 84 nM. ATP-induced oligomerization of FliI induced kinetic changes, stimulating fast-on, fast-off binding and lowering affinity. Full length FlhB purified under solubilizing, nondenaturing conditions formed a stable dimer via its transmembrane domain and stably bound FliH. Together, the present results support the previously hypothesized central role of FlhB and elucidate the dynamics of protein-protein interactions in type III secretion.

Introduction

The bacterial flagellum is a proton-driven rotary nanomachine responsible for motility in many species [1,2,3]. Most proteins that comprise a flagellum reside beyond the cytoplasmic membrane and must be secreted. Secretion occurs via a specialized type III secretion system (T3SS or “export apparatus”[4,5]). Utilizing protonmotive force [6,7,8], the apparatus
translocates flagellar proteins across the cytoplasmic membrane into the central channel within
the growing flagellum through which they transit to their final location [9]. Homologous
T3SSs effect many modes of bacterial pathogenesis using needle-like structures that closely
resemble flagella [10].

The core flagellar T3SS consists of three soluble proteins (FliH, FliI and FliJ) and six integral
membrane proteins (FlhA, FlhB, FliO, FliP, FliQ and FliR) that are housed within the mem-
brane-supramembrane ring of the basal body. Like most of the other proteins, FlhB is necessary
for secretion and is critical for the substrate specificity switching that occurs upon hook com-
pletion as the export apparatus shifts from rod and hook-type proteins to filament-type pro-
teins [4,11]. FlhB undergoes asparagine-mediated autohydrolysis at N269-P270 [12,13].
Mutations in FlhB that slow or abolish this cleavage give rise to dramatically altered flagellar
structures due to defects in switching [14]. FliK is one of the rod- and hook-type substrates rec-
ognized via the flagellar T3SS during hook assembly [15]. A specific interaction of FlhB with
FliK is central to the switch, in which the T3SS stops exporting rod and hook-type proteins and
begins exporting filament-type proteins [16,17] via a mechanism modeled as a “temporal tape
measure” in which FliK interacts with both FlhB and hook proteins [18,19]. The interaction
between FliK and FlhB is thought to vary as a function of hook length, though the details of
how remain unknown [20]. In addition to FliK, FlhB has been reported to bind FliH, FliI, FliJ
and perhaps the cytoplasmic domain of FlhA (FlhA_C) [21], though the veracity and conse-
quences of these interactions are largely unknown. Full-length FlhA and FlhB exhibited no
binding to each other in affinity blots [22].

While a great deal of work has described apparatus proteins with respect to requirements
for secretion, qualitative interactions and structure, understanding of dynamic interactions has
lagged. Kinetic relationships are fertile ground for exploration and characterizing them will
provide a better understanding of T3S and transmembrane transport in general. Much of what
is known about interactions among export proteins, substrates and chaperones derives from
copurification and affinity blotting experiments that have limitations such as requirements for
attainment of equilibrium and that only high affinity interactions can be observed. Using a
type of optical biosensing, biolayer interferometry (BLI) [23], and analytical ultracentrifugation,
the present study was able to address oligomerization of full-length, membrane integrated FlhB
as well as the complex kinetic interactions of FlhB and its cytoplasmic domain with other T3S
apparatus proteins.

Similar to surface plasmon resonance (SPR), BLI allows real-time measurement of protein-
protein interactions and determination of kinetic and affinity constants [24]. Ligand proteins
are tethered to fiber optic sensors and dipped into analyte-containing buffers to measure asso-
ciation. Dissociation is monitored after movement to buffer without analyte. Instrument
response, measured in nanometers of shift of the interference pattern of white light caused by
analyte-induced changes in the distance between two reflecting surfaces over time, yields asso-
ciation and dissociation rate information. Fits of raw data to kinetic models allow assignment
of rate and affinity constants. In the case of simple binding, fits to single exponentials allow
determination of k_{off} from the dissociation phase since reassociation is negligible due to dilu-
tion of dissociated analyte. Fitting the association phase yields observed rate constants (k_{obs}),
from which k_{on} can be extracted given analyte concentration and k_{off}.

We were able to characterize the mostly weak, complex interactions of FlhBC with FliK,
FlhA_C and FliI. Provision of ATP to FliI dramatically altered binding, weakening affinity. FlhB
was shown to form a stable dimer via the transmembrane domain and to bind FliH. The cur-
rent work not only sheds light on dynamic events in flagellar T3S, but also sets a foundation for
future studies utilizing the membrane proteins of the apparatus in optical biosensing.
Materials and Methods

Overexpression and purification

Plasmids used in this study are shown in S1 Table. His-tagged variants of the soluble export proteins and the cytoplasmic domains of FlhA and FlhB (“FlhAC” and “FlhBC”) were overproduced and purified. Overnight cultures of E. coli BL21DE3(pLysS) cells harboring plasmids encoding His-tagged proteins were subcultured and grown in Luria broth at 30°C to an OD600 ~ 0.4. Expression was induced by addition of 0.2 mM IPTG, after which growth was continued for four hours. Cells were harvested by centrifugation and pellets were frozen at -80°C until use.

All purification steps were performed on ice or at 4°C. Pellets from 1 L cultures were thawed and resuspended in 25 ml lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 0.1% Tween-20 and 200 μg ml⁻¹ lysozyme). Resuspended cells were passed through a French press at 20,000 psi and then subjected to centrifugation for 20 min at 10,000 × g at 4°C. The resulting clarified supernatant was transferred to a tube containing 1 ml of equilibrated Talon (BD Biosciences) immobilized metal affinity chromatography (IMAC) resin.

Batch binding was allowed to proceed with gentle agitation for 20 min after which the resin was pelleted by brief centrifugation and washed twice with 20 ml wash buffer (50 mM Tris pH 8.0, 500 mM NaCl, 25 mM imidazole, 0.1% Tween-20). The resin was transferred to a column and washed with an additional 10 ml. Elution was achieved by addition of elution buffer (wash buffer with 250 mM imidazole). Proteins were exchanged into HBS-T (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20) by gel filtration and used immediately, or glycerol was added to 10% and proteins were snap frozen in liquid nitrogen and stored at -80°C until use. Concentrations were determined by Bradford assay [25] using BSA as standard.

Full-length FlhB was overproduced and purified under nondenaturing conditions from solubilized crude membrane fractions as described for FlhA [22]. For AUC studies the uncleavable variant of full-length FlhB, FlhB(N269A), the method of Fleming et al. [26] was modified as follows: cells overexpressing FlhB(N269A) were resuspended in 10 mM phosphate buffer pH 8.0, 500 mM NaCl, 20% glycerol, 10 mM β-mercaptoethanol, 10 mM imidazole and lysed by sonication. Lysates were centrifuged at 10,000 × g to pellet unbroken cells. Supernatants were ultracentrifuged at 100,000 × g to pellet membranes. The crude membrane fraction was resuspended in lysis buffer (same as above but with 1% Thesit), homogenized and stirred at 4°C for 1 hour. After centrifugation at 100,000 × g for 45 min, the supernatant was retained as solubilized membrane fraction and subjected to IMAC to purify the FlhB(N269A). Wash and elution buffers were the same as the lysis except containing 20mM and 250 mM imidazole, respectively. To exchange the Thesit for E₈C₅, a detergent with the same partial specific volume as water and hence amenable to analytical ultracentrifugation, purified FlhB(N269A), ~25 ml, was diluted in 1 L dilution buffer (10 mM phosphate buffer, pH 8.0, 1% Thesit, 20% glycerol, 20mM β-mercaptoethanol and then loaded onto a 1 ml SP Sepharose column. The column was washed with 50 ml of 10 mM phosphate buffer, 33 mM C₅₈E₅, 10 mM NaCl at 1 ml min⁻¹. FlhB(N269A) was eluted in 1 ml fractions in the phosphate/C₅₈E₅ buffer with 500 mM NaCl.

Optical biosensing

All biolayer interferometry (BLI) measurements were made on a FortéBio (Menlo Park, CA) Octet QK biosensor using streptavidin (“SA”) sensors. Assays were performed in 96-well microplates at 25°C. All volumes were 200 μL. Ligand proteins were exchanged into HBS-T by passage over a desalting column. Biotinylation by amine crosslinking to NHS-LC-LC-biotin (succinimidyl-6-[biotinamido]-6-hexanamidohexanoate) was performed at a 5:1 molar ratio of
biotin to protein for 30 min at room temperature followed by separation of protein from free biotin by repeated passage over a desalting column. After loading ligands onto SA sensors, a baseline was established in buffer prior to association at varying analyte concentrations. Dissociation was subsequently measured in buffer only. All phases were done in HBS-T, except the full-length FlhB experiment, in which the Tween was replaced with 1% Triton X-100. Raw data were analyzed with GraphPad Prism.

For numerical simulation of FliK-FlhB\(_C\) binding, a conformational change model (A + B \rightleftharpoons AB \rightleftharpoons AB^{+}) was made in which A is analyte (FlhB\(_C\)), B is ligand, AB is the bound complex and AB\(^{+}\) is a conformationally altered state. Rate constants \(k_1\) and \(k_2\) govern association and dissociation of the free proteins and \(k_3\) and \(k_4\) describe the shift to and from the AB\(^{+}\) state, respectively. The set of differential equations used for the simulations were:

\[
\begin{align*}
\frac{dAB}{dt} &= k_1A \cdot B + k_2AB - k_3AB - k_4AB^+ \\
\frac{dA}{dt} &= k_2AB - k_1A \cdot B \\
\frac{dAB^+}{dt} &= k_3AB - k_4AB^+
\end{align*}
\]

Data were plotted as fractions of maximal binding (\(B_{max}\)), which was iteratively determined. A 15% correction factor to account for differences between the signal produced by AB\(^{+}\) relative to AB was included in the simulations.

### Analytical ultracentrifugation

Sedimentation equilibrium ultracentrifugation was performed using a Beckman Optima XL-A ultracentrifuge and an AnTi 60 rotor essentially as described [27], except that the buffer contained 33 mM C\(_8\)E\(_5\). Scans were collected at 280 nm with a spacing of 0.001 cm in the step mode with twenty averages per step. Three scans were superimposed prior to analysis with Optima XL-A/XLI version 4.0 (Beckman).

### Results

#### Biosensing Survey

To examine dynamic interactions between FlhB\(_C\) and other apparatus proteins, FlhB\(_C\) was used as analyte versus each of the other apparatus proteins as ligand (Fig 1A). A starkly
different shift profile from nonspecific control binding (Fig 1B, black trace) was observed for FliK. Smaller differences were noted for all other export proteins, e.g. a small amplitude fast on state for FlhAC, indicating some interaction with FlhBC. Observed binding was complex and nonspecific binding (NSB) as evidenced by response to BSA as ligand was in many cases significant. FliJ, FliH and FlhBC ligands also exhibited binding different from BSA, but were resistant to further analysis due to NSB, low signal and other reasons. Biotinylated FlhBC was tethered to SA sensors and screened for binding versus analyte soluble export apparatus proteins at 1 μM (Fig 1C). Differences in FliI binding were noted. FliK-FlhBC, FlhAC-FlhBC and FlhBC-FliI interactions were selected for further kinetic characterization.

Kinetic characterizations

We previously reported a KD of 3.2 μM for FliK-FlhBC binding determined by steady state analysis of SPR data [13]. Delving further into the complexity to better understand the kinetics, BLI sensorgrams were collected for a concentration course ranging from 0 to 5 μM FlhBC. As shown in Fig 2A and 2B, association and dissociation phases could be fit by two exponentials, i.e. parallel events, but there was no global solution that yielded constants that fit two independent states. Instead, numerical simulations were performed using differential equations constructed from a conformational change model. Simulations of association-then-dissociation are shown for 5, 4, 3, 2 and 1 μM in Fig 2C–2G. Parameters for constants used in the simulations are shown in Table 1 and include slow transitions to and from the AB/C3 state.

Plotting k1 determined from simulations, which is equivalent to the observed rate constant (kobs) for initial binding in that it also accounts for dissociation occurring during the association phase, vs. analyte concentration (Fig 2H) yielded k_on of 5.5 x 10^4 M^-1 s^-1. Combination with a k_off of 0.44 s^-1 gave a KD of 8.0 μM for the initial binding event, consistent with our earlier study. Supporting the conformational change model is the observation that the amplitude of the slow-off state in the dissociation phase varied proportionately with the length of the association phase (S2 Fig). It should be noted that amplitude variations between full kinetic characterizations and the Fig 1 survey are likely a function of different specific binding activities of different preparations for both ligand and analyte. All concentration courses in this experiment were done with dilutions of the same preparation. We also note that the overall KD determined by steady state analysis, i.e. including the slow states, for FliK-FlhBC in Fig 2 is 2.1 μM (S3 Fig), almost identical to that of the preparations used in the earlier report despite very different amplitudes.

Ligand FlhAC-analyte FlhBC binding also exhibited complexity and did not fit global one-state association-then-dissociation models. Single exponentials did fit the association phase (Fig 3A). Global two-state exponentials could fit dissociation with k_off of 0.13 s^-1 and 4.7 x 10^-3 s^-1 (Fig 3B). Saturation analysis (Fig 3C) yielded a KD of 1.1 μM. Plotting k_on vs [FlhBC] led to an estimate of k_on of 8.5 x 10^4 M^-1 s^-1 and thus a nominal one-state k_off of 0.09 s^-1, though caution should accompany interpretation of these values (see Discussion).

FlhB binds FliI via its cytoplasmic domain. As shown in Fig 4, ligand FlhBC bound FliI both in the absence and presence of Mg2+-ATP. For FliI without ATP (Fig 4A), fits to a global one-state model yielded a KD of 84 nM with a k_on of 1.8 x 10^4 M^-1 s^-1 and k_off of 1.5 x 10^-3 s^-1. Pre-incubation of analyte FliI with an excess of ATP resulted in near elimination of nonspecific binding and more complex kinetics; substantial fast-on and fast off-states are seen relative to the no ATP sample. Data do not fit two-state models, perhaps reflecting additional states induced by oligomerization of FliI (see Discussion). KD determined from steady state analysis (Fig 4C) is 1.1 μM.
Fig 2. Simulation of FliK-FlhB\textsubscript{C} binding. A and B, association and dissociation phases. Concentrations of FlhB\textsubscript{C} were 5, 4, 3, 2, 1, 0.5, and 0 \( \mu \text{M} \). The 0.5 and 0.25 \( \mu \text{M} \) traces are unlabeled. Fits to individual two-state exponentials are shown as red lines. C-G, Simulations of the 5, 4, 3, 2 and 1 \( \mu \text{M} \) data with a conformational change model using global parameters for \( \text{k}_{\text{off}} \) and \( \text{k}_{\text{on}} \) and \( \text{k}_{\text{off}} \) for transition to the conformationally altered state (Table 2). H, Apparent \( \text{k}_{\text{on}} \) vs [FlhB\textsubscript{C}] to determine the global \( \text{k}_{\text{on}} \) (=slope).

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Full-length FlhB interactions

FlhBC–FlhBC interactions (Fig 1) were at best minimally observable, consistent with earlier studies that found questionable or no interaction [12,21]. We report here purification of solubilized FlhB under non-denaturing conditions using a procedure modified from a prior method used to purify FlhA [22]. The uncleavable but export competent N269A variant [14] was used to assure retention of the carboxyl-terminal subdomain in the solubilizing conditions used.

Table 1. Parameters determined by simulation of FliK-FlhB\(_C\) binding.

<table>
<thead>
<tr>
<th>[FlhBC], (\mu)M</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_1) (s(^{-1}))</td>
<td>0.25</td>
<td>0.25</td>
<td>0.2</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>(k_2) (s(^{-1}))</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
</tr>
<tr>
<td>(k_3) (s(^{-1}))</td>
<td>0.0085</td>
<td>0.0085</td>
<td>0.0085</td>
<td>0.0085</td>
<td>0.0085</td>
</tr>
<tr>
<td>(k_4) (s(^{-1}))</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>B(_{\text{max}})</td>
<td>1.7549</td>
<td>1.7549</td>
<td>1.7549</td>
<td>1.7549</td>
<td>1.7549</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0134884.t001

Fig 3. FlhAC-FlhB\(_C\) binding. Ligand FlhAC was exposed to 2, 1, 0.5, 0.25 and 0.125 \(\mu\)M FlhB\(_C\). A, association with fits to a one-state model B, dissociation with fits to a global two-state model C, steady state analysis. D, \(k_{\text{obs}}\) vs. [FlhB\(_C\)] to estimate kinetic constants, \(R^2 = 0.98\).

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though later purification of wild-type FlhB from pMM9, which complements a flhB null, showed that the subdomain consisting of residues 270–383 is retained (S1 Fig)). Anti-FlhB immunoblots of hook-basal bodies (HBBs) prepared from SJW880 [28] under conditions in which the C ring and export apparatus proteins are retained [29] (gift from Noreen R. Francis) demonstrated significant SDS-stable dimerization, as did purified FlhB(N269A) (Fig 5A). Full-length FlhB(N269A), solubilized in the neutrally buoyant, nondenaturing detergent C8E5, formed a stable dimer in a sedimentation equilibrium ultracentrifugation experiment (Fig 5B). Fits to a single species model produced a molecular weight of 84.1 kDa, consistent with a FlhB dimer. The tagged monomer is ~42.3 kDa.

Purified wild-type FlhB exhibited specific binding to FliH as ligand. Dissociation anomalies perhaps due to detergent effects prevented kinetic analysis. $K_D$ determined from steady state analysis (Fig 6) was 0.8 μM. The 2 μM sample was excluded from analysis due to anomalous readings from that channel, though its inclusion would render a $K_D$ of 0.9 μM with a concomitant reduction in $R^2$ from 0.99 to 0.82. Further experiments with other analytes were precluded by instability of the FlhB preparations; we hope to examine them in future studies.

In summary, the present results assign rate and affinity constants to binding of FlhB to several apparatus proteins and provide mechanistic clues for T3S. They are consistent with a conformational change in FlhB upon FliK binding and ATP-induced kinetic alterations in FlhB-FliI interactions as well as weak FlhB-FlhA cytoplasmic domain interactions and FliH interactions with full length FlhB.

### Table 2. Kinetic constants determined from BLI experiments for binding to FlhBC

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$K_D$</th>
<th>$k_\text{on}$ (M$^{-1}\text{s}^{-1}$)</th>
<th>$k_\text{off}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlhAc</td>
<td>1.0 μM</td>
<td>8.5 x 10$^4$</td>
<td>0.085</td>
</tr>
<tr>
<td>FliI (-ATP)</td>
<td>84 nM</td>
<td>1.8 x 10$^4$</td>
<td>1.5 x 10$^{-3}$</td>
</tr>
<tr>
<td>FliI (+ATP)</td>
<td>1.1 μM</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FliK</td>
<td>8.0 μM</td>
<td>5.5 x 10$^4$</td>
<td>0.44</td>
</tr>
</tbody>
</table>

ND, not determined. Constants are expressed with respect to the monomer concentrations.

doi:10.1371/journal.pone.0134884.t002
Discussion

Perhaps the best information about protein-protein interactions in the flagellar T3SS originates from intergenic suppression studies, e.g. [16,30]. However, much of the current conception of these interactions is based on qualitative equilibrium methods such as immunoaffinity blotting and copurification that can effectively identify only strong interactions [12,21,22,31]. These methods can also make interpretation difficult due to NSB. NSB was certainly a challenge in the present study and may be an inherent consequence of examining pairwise interactions

Fig 5. Full-length FlhB forms a dimer. A, anti-FlhB immunoblot of hook-basal body preparation (HBB) and purified FlhB(N269A). Approximate locations of molecular weight standards in kDa are shown at left. B, sedimentation equilibrium analytical centrifugation. A fit is shown to a single-species model, the molecular weight of which is 84.1 kDa (monomer of tagged FlhB(N269A) = 42.3 kDa).

doi:10.1371/journal.pone.0134884.g005
between proteins whose normal environment is within a membrane-integrated macromolecular complex. We were able to measure NSB via the proxy of BSA binding, performing full analyses only when it was not a substantial fraction of total binding.

BLI of course has interpretative limitations as well. Since sensors are coated in ligand, sensors without ligands are not true references in that they can present a surface that differs in electrostatic and other properties. Use of BSA as a non-related ligand presents similar concerns, though binding of export proteins to both BSA and sensors without ligand was similar (data not shown). Ligand activity and presentation upon biotinylation could explain failure to observe binding on reversal of ligand-analyte pairings.

Interestingly, significant binding was not observed with several interactions previously reported. While present results cannot rule out interactions not observed, it is possible that at lower concentrations than those used in affinity blotting, FlhB engages in a different set of interactions. For example, ligand FlhB<sub>C</sub> bound only FliI and perhaps FlhB<sub>C</sub>, exhibiting essentially no affinity for other apparatus proteins even though positive in affinity blotting [21]. Another possibility is that interactions may be biologically relevant even though they associate slowly but are stable once formed. The weaker interactions (FliK-FlhB<sub>C</sub>, FlhB<sub>C</sub>-FliI (with ATP), and FlhAC-FlhB<sub>C</sub>) are the ones more likely to undergo dynamic changes during export as they exhibit significant off rates.

All of the characterized interactions exhibited more than simple one-state binding. Whether the secondary events are biologically relevant is an open question. We suspect the initial, fast

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**Fig 6. Steady state analysis of FliH binding to full length wild-type FlhB.** Association phases from which steady state amplitudes were determined are shown in the inset. FliH concentrations ranged from 0.125 to 8 μM.

doi:10.1371/journal.pone.0134884.g006
but low affinity events are the relevant ones and that the slower on and off events may be due to aggregation, surface-associated denaturation or other biologically irrelevant events. Surface-associated denaturation in BLI has been observed for calmodulin-nitric oxide synthase [32] and *Helicobacter pylori* UreE-UreG binding [33]. A potential example in this study is the slow-off FlhAC-FlhBC dissociation phase (Fig 3B).

Kinetic and affinity constants determined with varying degrees of veracity are shown in Table 2.

**FliK-FlhBC interactions**

Though known to interact for many years, binding between FliK and FlhBC was first directly observed using biosensing [13]. The one-state with conformational change model described herein fits observed FlhB-FliK binding. Affinity for the initial binding and dissociation is 8 \( \mu \text{M} \), in good agreement with the previous report, which was determined by saturation binding and reflects both states. The fast-on, fast-off initial binding is consistent with intergenic suppression data and failure to observe interactions by equilibrium methods. Slow transitions to and from the conformationally altered state (\( AB^* \), see Results) render it a minor event, but one sufficient to explain the complexity observed. Whether it is a biologically relevant state and whether it changes in the presence of other proteins, e.g. substrates, or structural changes brought about by hook completion, remains a subject for further investigation, though conformational flexibility appears to be important for FlhB function [34]. Alterations in the dynamics of the conformational change would also be consistent with the temporal tape measure model.

**FlhAC-FlhBC interactions**

Compared to the NSB indicated by FlhBC binding to BSA sensors (Fig 1B), FlhAC-FlhBC binding exhibited fast on and fast off components (Fig 3). The most likely interpretation is that these proteins possess weak affinity for one another and that the slow-off state represents an irreversible, biologically irrelevant state. Conversely, it may signal a conformationally changed, high affinity state, but qualitative evidence suggesting weaker [21] or undetectable [22] binding supports the former interpretation. FlhAC-FlhBC interactions are depicted as gating the membrane pore, e.g. [35]. Weak binding in the absence of the transmembrane domains may be exemplary of this.

**FlhB-FliI Binding**

ATP induces hexamerization in FliI [36,37]. The present results show that in addition to inducing oligomerization, ATP alters FliI interactions with FlhBC. Presumably monomeric FliI without ATP shows tight binding to FlhB with relatively low NSB (Fig 4A). Addition of an excess of ATP resulted in substantially lower affinity (Fig 4C) but faster association and dissociation and additional complexity consistent with the hypothesis that FliI undergoes repeated binding and release events in delivering export-competent substrates to the export gate [38] and recent observations of FliI turnover in the basal body [39].

**FlhB dimerization and interaction with FliH**

Ferris et al. extensively searched for FlhBC-FlhBC interactions, finding none using equilibrium methods [12]. Our BLI data, too, indicate very little interaction between the cytoplasmic domains, though there may be some low affinity binding. Indeed, one interpretation of the complexity observed in BLI of FliK-FlhBC binding was oligomerization of FlhBC [13]. Development of a purification scheme for native, full-length FlhB allowed us to show that it forms a
stable dimer in detergent micelles. Additional evidence from the HBBs suggests that FlhB forms dimers in vivo as well. The high affinity of the transmembrane domain-containing FlhB and the extremely low affinity (if present at all) of the cytoplasmic domains for themselves may hint at the dynamics of FlhB; the proximity of cytoplasmic domains forced by dimerization of the transmembrane domains may facilitate otherwise weak binding that may undergo cycles of association and dissociation as secretion occurs.

FliH exhibited significant binding only to full-length FlhB (Fig 6). Two possibilities suggest themselves: the binding site may reside at least partly within the transmembrane domain of FlhB; or FliH may be active with respect to FlhB binding in detergent micelles, which can be considered unsurprising as it partitions with the membrane even in the absence of basal bodies [40]. Other apparatus proteins were not investigated in this study with respect to binding the full-length FlhB due to the difficulty of the purification and the loss of FlhB binding activity over time. We hope to characterize these events in a future study.

The present results expand knowledge of the dynamic interactions of FlhB with other export apparatus proteins and assign rate and affinity constants to them. In short, FlhB stably dimerizes and stably binds FliH; FlhBC binds FliK and FlhAC with micromolar affinity and complex kinetics. Interactions with FliI shift upon addition of ATP, lowering affinity but increasing the rates of association and dissociation. The complexities observed underlie the mechanism of T3S. How interactions change when they are more than pairwise, as in vivo, is an active area of investigation.

Supporting Information

S1 Fig. Purification of full length FlhB. A, Coomassie stained SDS-PAGE of samples taken during purification. Lanes are: 1, uninduced cells; 2, induced cells; 3, crude lysate; 4, 1st low-speed supernatant (clarified lysate); 5, 1st high-speed supernatant; 6, 1st high-speed pellet (crude membranes); 7, solubilization (overnight); 8, second high-speed pellet; 9, solubilized sample (load); 10, flow-through 11; pooled first wash; 12, final wash; E1–5, eluted protein fractions 1–5. B, Immunoblotting analysis of uninduced (U), induced (I) and purified (E2, diluted 10x), with positions of FlhBTM+CN and FlhBCC denoted with arrowheads at right. Note that anti-His only responds to FlhBTM+CN as the His-tag is amino-terminal, anti-FLAG is overexposed and anti-FlhB is more reactive to FlhBCC, as has been noted previously (31). (JPG)

S2 Fig. Slow-off state increases in amplitude as a function of association time. Ligand FliK was exposed to analyte 2 μM FlhBC for various times of association after which dissociation was monitored for 300 s. Time of association was 10 s (green), 30 s (yellow), 60 s (orange), 180 s (blue), and 900 s (brown). (TIF)

S3 Fig. Slow-off state increases in amplitude as a function of association time. Ligand FliK was exposed to analyte 2 μM FlhBC for various times of association after which dissociation was monitored for 300 s. Time of association was 10 s (green), 30 s (yellow), 60 s (orange), 180 s (blue), and 900 s (brown). (TIF)

S1 Table. Plasmids used in this study. (PDF)
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Author Contributions

Conceived and designed the experiments: JLM TM KN. Performed the experiments: JLM YF JWF SAH KAH. Analyzed the data: JLM TM YF KN JWF SAH KAH. Contributed reagents/materials/analysis tools: JLM TM YF KN JWF SAH KAH. Wrote the paper: JLM TM KN JWF SAH KH.

References


