

RESEARCH ARTICLE

Hook-basal-body assembly state dictates substrate specificity of the flagellar type-III secretion system

Alina Guse ^{1,2} | Manuel Halte ¹ | Svenja Hüsing ¹ | Marc Erhardt ^{1,3}

¹Institute for Biology, Humboldt-Universität zu Berlin, Berlin, Germany

²Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, USA

³Max Planck Unit for the Science of Pathogens, Berlin, Germany

Correspondence

Marc Erhardt, Institute for Biology, Humboldt-Universität zu Berlin, Philippstr. 13, 10115 Berlin, Germany.
Email: marc.erhardt@hu-berlin.de

Funding information

H2020 European Research Council, Grant/Award Number: 864971; Deutsche Forschungsgemeinschaft, Grant/Award Number: ER 778/2-1; Humboldt-Universität zu Berlin

Abstract

The assembly of the bacterial flagellum is orchestrated by the secretion of distinct early and late secretion substrates via the flagellar-specific type-III secretion system (fT3SS). However, how the fT3SS is able to distinguish between the different (early and late) substrate classes during flagellar assembly remains poorly understood. In this study, we investigated the substrate selectivity and specificity of the fT3SS of *Salmonella enterica* at different assembly stages. For this, we developed an experimental setup that allowed us to synchronize hook-basal-body assembly and to monitor early and late substrate secretion of fT3SSs operating in either early or late secretion mode, respectively. Our results demonstrate that the fT3SS features a remarkable specificity for only the substrates required at the respective assembly stage. No crosstalk of substrates was observed for fT3SSs operating in the opposing secretion mode. We further found that a substantial fraction of fT3SS surprisingly remained in early secretion mode. Our results thus suggest that the secretion substrate specificity switch of the fT3SS is unidirectional and irreversible. The developed secretion substrate reporter system further provides a platform for future investigations of the underlying molecular mechanisms of the elusive substrate recognition of the T3SS.

KEYWORDS

flagella biosynthesis, motility, protein transport, substrate specificity switching, type-III secretion

1 | INTRODUCTION

Many bacteria move in liquid environments using a macromolecular propeller, the flagellum. A type-III secretion system (T3SS) at the base of the flagellum and the related injectisome of Gram-negative pathogens are required to build and operate these sophisticated nanomachines. The flagellar structure can be divided into three main parts:

(a) a cell envelope-anchored basal body (consisting of the C-ring, the flagellar-specific T3SS, the MS-ring, the rod and the LP-ring), (b) a hook and, (c) a filament. Secretion of a variety of different T3SS substrate proteins occurs from the cytoplasm to the distal end of the growing structure in a highly regulated and sequential manner (Chevance & Hughes, 2008; Diepold & Wagner, 2014; Wagner et al., 2018). The different secretion substrates of the flagellar T3SS (fT3SS) have been

Manuel Halte and Svenja Hüsing are equal contributors.

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classified into two classes: early (rod/hook-type proteins) and late (filament-type proteins) (Minamino & Macnab, 1999). The σ^{54} -dependent transcription factor σ^{54} is composed of five core components (FliPQR, FliHBA) and an associated cytoplasmic ATPase complex (FliHIJ). FliPQR and FliHBA form a unique helical pore complex that is primarily located within the periplasm above the inner membrane and conducts unfolded proteins into the narrow secretion channel (Johnson et al., 2019; Kuhlen et al., 2018, 2020). The transmembrane domains of FliA surround the pore complex in the inner membrane, while the large C-terminal domains of nine FliA subunits form a nonameric ring underneath the pore in the cytoplasm (Abrusci et al., 2012; Terahara et al., 2018). FliH is the so-called switch protein of the σ^{54} -dependent transcription factor that is involved in substrate specificity switching from early to late secretion substrates according to the flagellar assembly state. Substrate specificity switching was shown to require autocatalytic cleavage of the large C-terminal domain of FliH (FliH_C) at a highly conserved NPTH motif into the subdomains FliH_{CN} and FliH_{CC} (Ferris et al., 2005; Fraser et al., 2003; Minamino & Macnab, 2000; Monjarás Feria et al., 2015; Sorg et al., 2007; Zarivach et al., 2008). An autocleavage-deficient mutant (FliH_{N269A}) is unable to secrete late secretion substrates (filament-type proteins) and hook-length is not controlled, resulting in the formation of polyhooks (Fraser et al., 2003). The switch in substrate specificity is induced after hook assembly is completed, presumably by an interaction of the molecular ruler protein FliK with FliH_C. When the hook reaches its final length of approximately 55 nm in *Salmonella*, FliK resides temporarily in the secretion channel through brief interactions between its N-terminal domain and the hook subunits (FliG) and the hook cap (FliD) (Minamino et al., 2009; Moriya et al., 2006). This pause in secretion places the C-terminal domain of FliK in close proximity to cleaved FliH_C, thereby allowing the interaction between the two domains and subsequent conformational rearrangements of the substrate docking platform to render the secretion apparatus competent for late substrate secretion (Ferris & Minamino, 2006; Kinoshita et al., 2017, 2020; Minamino, 2018; Minamino et al., 2020).

Here, we aimed to investigate the remarkable selectivity and specificity of the σ^{54} -dependent transcription factor in respect to early and late substrate recognition and secretion before and after hook completion. We therefore developed a synchronized secretion reporter platform that allowed us to analyze the substrate secretion profile of σ^{54} -dependent transcription factors operating in early (before hook completion) or late secretion mode (after hook completion). We found that the σ^{54} -dependent transcription factor specifically secretes only the substrates required for HBB assembly either before and after hook completion, respectively. These results suggest that the σ^{54} -dependent transcription factor secretion substrate specificity switch is unidirectional.

2 | RESULTS

2.1 | Establishment of a synchronized secretion reporter system

Flagellar assembly is tightly regulated and organized in a spatiotemporal manner, yet, assembly of multiple flagella in a single cell (e.g. in

case of peritrichously flagellated bacteria such as *Salmonella*) is not synchronized (Chevance & Hughes, 2008; Macnab, 2003). A single *Salmonella enterica* (*S. enterica*) cell assembles on average 4–8 flagella (Aldridge & Hughes, 2002). Since the various flagella are in different assembly stages, the individual σ^{54} -dependent transcription factors have to select the correct substrate proteins that are required at this particular stage of assembly out of the large pool of cytoplasmic proteins (Figure S1a,b). Thus, each individual σ^{54} -dependent transcription factor is able to discriminate between different substrate types (i.e. early and late) that are needed at different stages of its assembly (Macnab, 2003). We hypothesized that the substrate specificity of the σ^{54} -dependent transcription factor could be explained by two distinct mechanisms. First, the σ^{54} -dependent transcription factor might be able to switch its substrate recognition ability (i.e. its substrate specificity) between the different substrate types either reversibly or irreversibly. Second, the σ^{54} -dependent transcription factor might simply start with the ability to recognize and secrete early-type substrates (i.e. rod/hook-type) and subsequently, after termination of hook growth, enlarge its substrate recognition spectrum to also include late substrates (i.e. filament-type) in addition to the early substrates (rod/hook-type). In order to investigate these possibilities, we developed an experimental setup that allowed us to synchronize hook-basal-body (HBB) assembly at various stages, corresponding to σ^{54} -dependent transcription factors operating in early or late mode. This allowed us to monitor (early and late) substrate secretion in a homogenous population (Figure S1c) in contrast to previous studies (Hirano et al., 2003; Stafford et al., 2007). We defined the early mode as secretion of substrates during assembly of the HBB structure, and the late mode as secretion of substrates after HBB completion and substrate specificity switching of the σ^{54} -dependent transcription factor. In order to obtain a homogenous population of HBBs with their σ^{54} -dependent transcription factors operating in the early secretion mode, we utilized a hook-deletion mutant strain (Δ fliG). A hook-deficient mutant is unable to induce the substrate specificity switch, which is thought to be mediated by an interaction between the molecular ruler FliK and the switch protein of the σ^{54} -dependent transcription factor, FliH, upon hook completion (Ferris et al., 2005; Hirano et al., 1994; Williams et al., 1996).

The main challenge in obtaining a homogenous population of cells with all their σ^{54} -dependent transcription factors operating in late secretion mode lies within the fact that conditions would need to be found, where all flagella would have completed HBB formation (and therefore have concluded the switch from early to late secretion mode). To overcome this problem, we synchronized flagellar assembly by placing the flagellar master regulator *fliHDC* under control of an anhydrotetracycline (AnTc)-inducible promoter (P_{tetA} -*fliHDC*) (Karlinsky et al., 2000). We induced *fliHDC* expression by addition of AnTc for 120 min, before we removed the inducer and continued bacterial growth for 90 min (pulse *fliHDC* induction). We reasoned that this procedure allowed for enough time to complete HBB assembly of all initiated flagellar complexes (and, presumably, allow enough time for the substrate specificity switching to occur in all σ^{54} -dependent transcription factors). To verify HBB completion and the switch in substrate specificity, we visualized assembled hooks (Figure 1), or hooks and attached filaments simultaneously (Figure 2). We chose to label the hook as a marker for completed HBB assembly as it is the first structure protruding from the cell envelope during

flagellar assembly. In order to determine the number of hooks that were assembled during a specific timeframe post induction of *flhDC*, we utilized a mutant of the hook subunit FlgE that harbored a surface-exposed, accessible cysteine substitution (FlgE_{S171C}). This enabled us to stain the hook with different dyes at various times of the experiment using fluorophore-coupled maleimide dyes. Since an individual hook is made of ~120 subunits of FlgE (Macnab, 2003), binding of the maleimide dye resulted in quantifiable fluorescent foci. During the initial 120 min pulse induction of *flhDC* (FlhDC+), we labeled the hooks with DyLight™ 550 Maleimide (t_1 , Alexa550) (Figure 1a). After removal of the inducer, the cells were further incubated for 90 min to allow for HBB completion of all initiated complexes, and any newly

formed hooks were masked by addition of Maleimide-PEG₂-Biotin (Biotin-Mal). We masked with Biotin-Mal to be able to distinguish between hooks that assembled during *flhDC* induction and those that assembled after the time given for HBB completion. Finally, we labeled hooks that formed 90 min after removing the inducer with DyLight™ 488 Maleimide (t_2 , Alexa488). We reasoned that, if no new HBBs were detected after the final Alexa488 labeling, all HBBs in the analyzed population of cells would have had enough time to reach an identical assembly state during the 90 min incubation after inducer removal. To demonstrate the difference between synchronized and nonsynchronized HBB assembly, we continuously added AnTc to a control sample, resulting in constant induction of *flhDC* (Figure 1b).

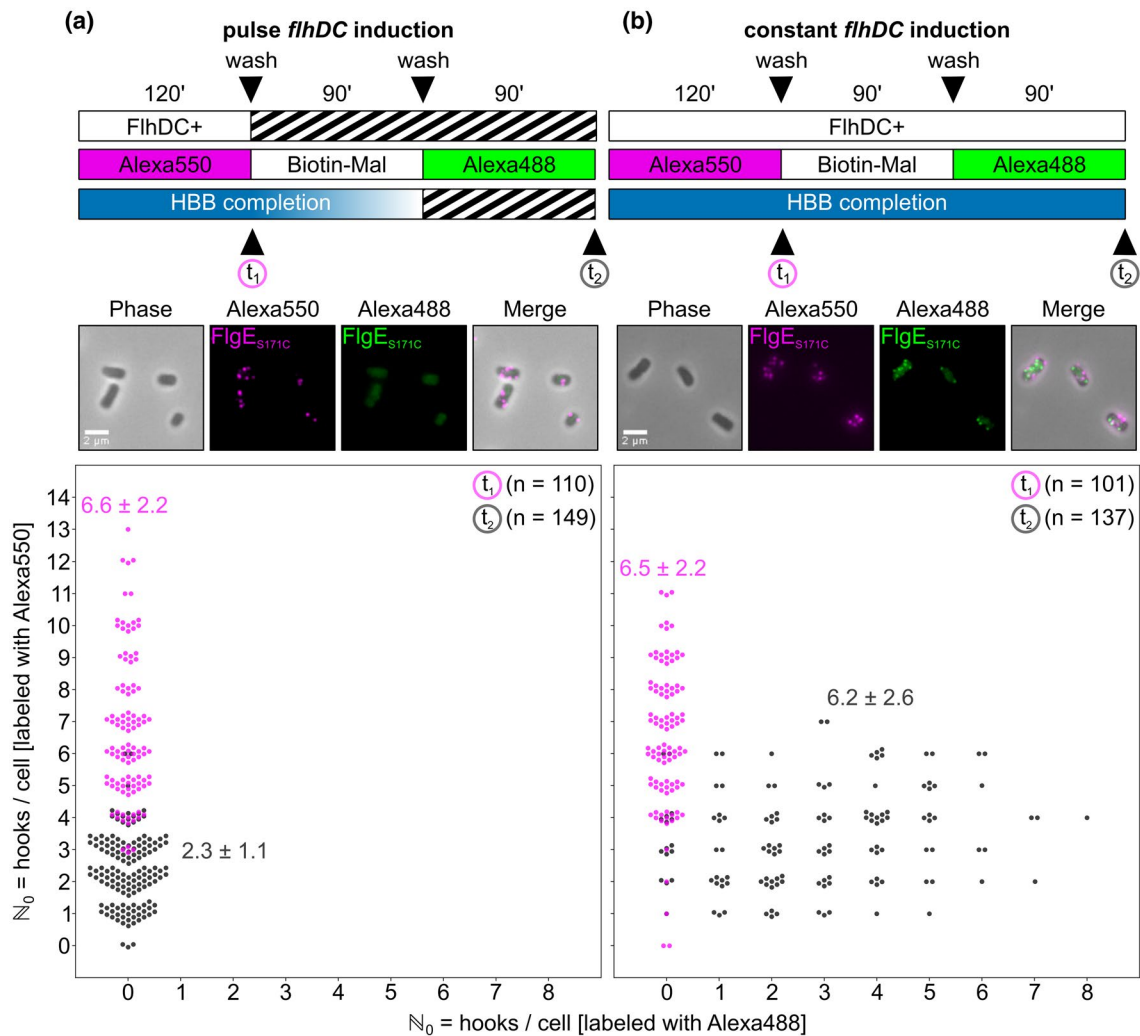


FIGURE 1 Development of an experimental setup to synchronize flagellar assembly. The reporter strain harbors an inducible flagellar master regulator (P_{tetA} -*flhDC*) and a surface-exposed, accessible cysteine residue within the hook subunit (FlgE_{S171C}) for labeling with fluorophore-coupled maleimide dyes. FlhDC expression was pulse-induced for 2 hr (a) or constantly induced (b). Top panels: Schematic of the experimental timeline indicating the induction time of FlhDC (FlhDC+), washing steps, the presumed time of hook-basal-body (HBB) completion, and the dyes used as well as the timepoints (t_1 , t_2) of sample acquisition for quantification of hooks. Middle panels: Representative fluorescence microscopy images of samples acquired at t_2 . We note that for pulse *flhDC* induction, a general cytosolic background fluorescence is observed in the Alexa488 channel. Scale = 2 μm. Bottom panels: Quantification of the number of hooks per cell (N_0) at t_1 (magenta) and t_2 (grey) labeled with DyLight™ 550 Maleimide (Alexa550) and DyLight™ 488 Maleimide (Alexa488), respectively. Single cells are represented by individual data points (the number of analyzed cells are for pulse FlhDC induction; t_1 , $n = 110$, t_2 , $n = 149$ and for constant FlhDC induction; t_1 , $n = 101$, t_2 , $n = 137$). The average number of hooks per cell \pm SD per timepoint is indicated. SD, standard deviation

Fluorescent foci of labeled hooks were quantified after *flhDC* induction (t_1 , hooks labeled with Alexa555) and after HBB completion (t_2 , hooks additionally labeled with Alexa488) (Figure 1). On average, individual cells displayed 6.6 ± 2.2 (pulse AnTc induction) and 6.5 ± 2.2 (constant AnTc induction) hooks at time point t_1 , that is 120 min after the initial induction of *flhDC*. We next compared the number of HBBs per cell at t_2 for samples where we used pulse or constant induction of *flhDC*. We observed a substantial reduction in the number of hooks per cell (2.3 ± 1.1) for pulse-induced cells at t_2 , presumably because the cells continued to divide after inducer removal but stopped the synthesis of new HBBs (Figure 1a). In contrast, we observed on average 6.2 ± 2.6 hooks per cell at t_2 under conditions of constant induction of *flhDC*, which demonstrates that the cells continuously produced new HBBs during the duration of the experiment (Figure 1b).

Importantly, for pulse-induced cells we were unable to detect any newly synthesized hooks 90 min after inducer removal (t_2 , hooks additionally labeled with Alexa488). This suggested that incubation

for 90 min after removing the inducer of *flhDC* expression allowed for enough time to complete assembly of all previously initiated HBB complexes. We note that HBBs that completed assembly during the 90 min incubation period could not be included in the analysis because of the addition of Biotin–Mal. However, control results indicate that the total number of hooks per cell did not differ substantially when masking was not performed (Figure S2). For pulse-induced cells, only about 10% newly labeled HBBs were detected 90 min after inducer removal (t_2 , hooks additionally labeled with Alexa488) (Figure S2a). This suggested that only a minor fraction of HBBs initiated and completed assembly during the Biotin–Mal masking time-frame. These results are consistent with the previously reported short half-life of the FlhDC complex (Claret & Hughes, 2000) and verified that we were able to control the number of hooks per cell by controlling transcription of *flhDC*. However, we note that we might underestimate the total number of HBBs due to restraints given by the diffraction limit of the used microscope.

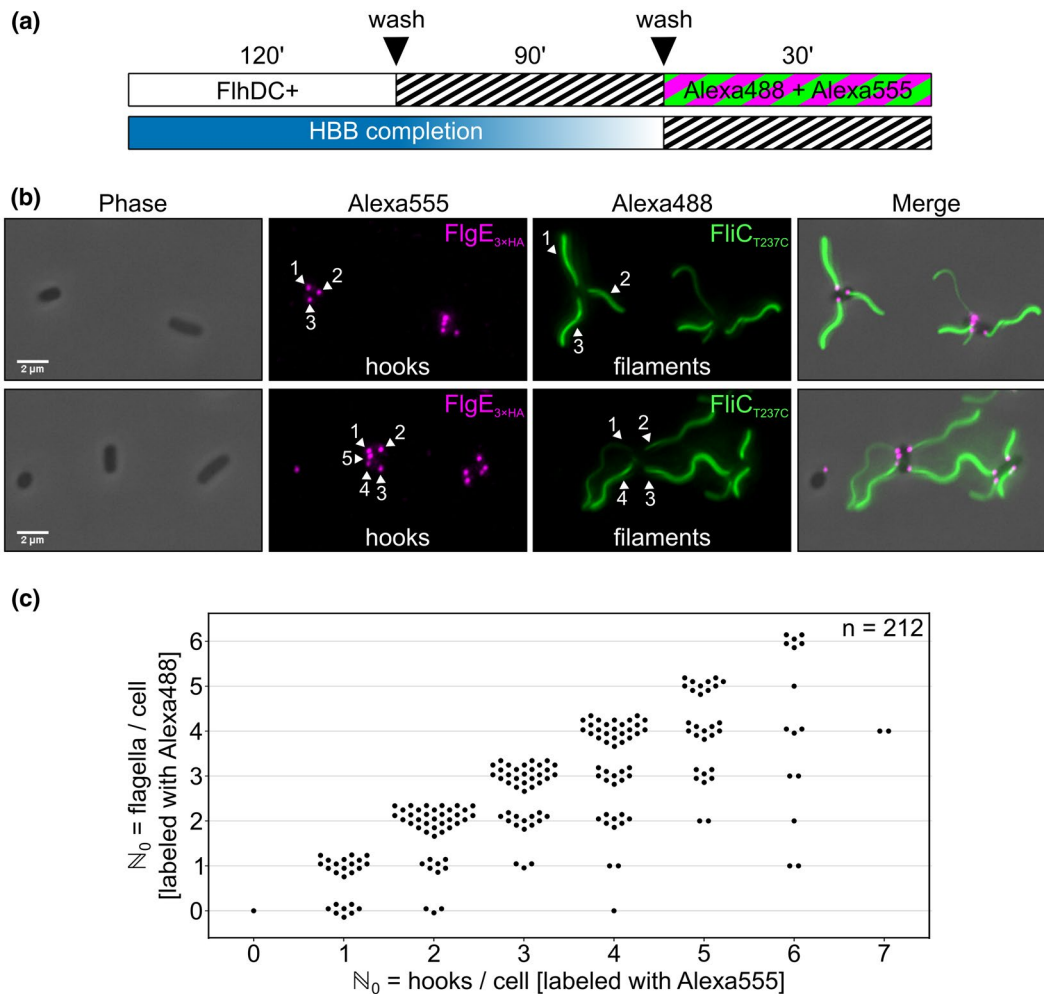


FIGURE 2 A sub-population of hook-basal-bodies is defective in filament assembly. Simultaneous immunostaining of hooks ($\text{FlgE}_{3 \times \text{HA}}$) and labeling of filaments ($\text{FliC}_{\text{T}237\text{C}}$) with fluorophore-coupled maleimide dye. (a) Schematic of the experimental timeline indicating the time of FlhDC induction ($P_{\text{tetA}}\text{-flhDC}$; FlhDC+), washing steps, the presumed time of hook-basal-body (HBB) completion and the used dyes. (b) Representative fluorescent microscopy images of bacteria with equal (top) or unequal (bottom) numbers of hooks and filaments. (c) Quantification of the number (N_0) of hooks (Alexa555) and filaments (Alexa488) per cell. Single cells are represented by individual data points ($n = 212$)

Next, we examined whether all HBB complexes had performed the switch to late secretion mode (i.e. able to secrete filament-type substrates). Therefore, we simultaneously visualized epitope-tagged hook subunits (FlgE_{3xHA}) using anti-HA immunostaining (anti-HA-Alexa Fluor 555 [Alexa555]) and the filament (FliC_{T237C}) using a fluorophore-coupled maleimide dye (DyLight™ 488 Maleimide [Alexa488]), respectively. Negative effects of the HA epitope tag on hook polymerization were excluded by performing swimming motility assays and monitoring the switch from early to late substrate secretion using a previously described Class 3 promoter reporter plasmid ($P_{motA-luxCDABE}$) (Figure S3a,b) (Brown et al., 2008; Goodier & Ahmer, 2001). We reasoned that all HBBs, which had successfully switched to late secretion mode would have a filament attached, while those that remained in early secretion mode would have no filament attached. We utilized the same experimental setup as outlined above for Figure 1, but performed the labeling only after the time given for HBB completion (Figure 2a). We observed a surprisingly large fraction of hooks (21%) without attached filaments (Figure 2b,c). No unattached filaments were observed, which likely excludes mechanical shearing of the flagellar filaments. To support our result, we performed intentional mechanical shearing of filaments by passing the culture multiple times through a syringe needle (Renault et al., 2017; Turner et al., 2012) before labeling the hook and filament in order to evaluate the frequency of hook detachment. We found that the ratio of hook-attached filaments and a filamentous hooks was independent of the induced shearing force (Figure S3c). We thus concluded that a certain fraction of HBBs is defective in late substrate secretion for unknown reasons and are therefore unable to polymerize filaments. However, because no new HBBs were assembled after the time given for HBB completion and the majority of HBBs had switched to late secretion mode, we decided to employ this experimental setup in order to investigate the substrate specificity of fT3SS operating in early or late secretion modes.

2.2 | The fT3SS is highly specific in substrate recognition and secretion

The above-described setup allowed us to analyze next which substrates are recognized and secreted by the fT3SS operating in either early or late secretion mode. In order to investigate the secretion of early and late substrates, we used reporter strains with the following main properties: (a) the flagellar master regulator was placed under an AnTc-inducible promoter ($P_{tetA-flhDC}$) to facilitate synchronization of fT3SS assembly as described above; (b) a deletion of the hook (early mode, $\Delta flgE$) or the hook-filament junction (late mode, $\Delta flgKL$) in order to ensure similar secretion levels within the synchronized population (we reasoned that filament assembly might slow-down the secretion of late substrates); and (c) individual early and late substrates were induced from a chromosomally encoded arabinose-inducible promoter (P_{araB} -substrate) to uncouple each substrate from the transcriptional hierarchy and to increase substrate expression, which facilitated detection of secreted substrate protein by Western

blot analysis (Figure 3a and Figure S1c). After pulse *flhDC* induction for 120 min (FlhDC+) and the HBB completion period of 90 min, we washed the cells again to remove all substrates that had been secreted into the supernatant up to this point (Figure 3b). Afterwards, we induced expression of individual early or late substrates by addition of 0.2% arabinose (ara; substrate+) and acquired samples after 0, 30, 60, and 90 min to monitor their secretion profile over time. As expected, early rod/hook-type substrates (we analyzed as representative substrates the hook cap protein FlgD and the molecular ruler protein FliK) were predominantly secreted in strains locked in the early secretion mode, but surprisingly, also to some extent in strains operating in the late secretion mode (Figure 3c, left panel). In contrast, late filament-type substrates (we analyzed the hook-filament junction protein FlgK and the filament cap protein FliD as representative substrates) were exclusively secreted under late secretion mode conditions (Figure 3c, right panel).

These observations suggest that the fT3SS enlarges its substrate recognition spectrum upon switching from early to late secretion mode (while presumably being able to continuously secrete early substrates in addition to late substrates, although early substrate secretion was substantially reduced). However, we note that we observed a minority of hooks without attached filaments in the previous fluorescent labeling experiment (Figure 2b,c), which might also account for the minimal secretion of early substrates in late secretion mode fT3SSs.

We therefore designed an experimental setup that allowed us to specifically block secretion through late-mode fT3SSs by plugging their secretion pore. To this end, we introduced a FliC-GFP fusion protein into our strains of interest. It had previously been shown that the T3SS is incapable of unfolding GFP, and thus GFP fusions to secretion substrates are not secreted (Akeda & Galán, 2005; Singer et al., 2014). The late substrate and filament subunit FliC on the other hand is unfolded and injected into the secretion channel by the fT3SS. We reasoned that a FliC-GFP fusion protein would therefore allow us to plug the secretion pore only in fT3SSs which had switched their substrate specificity to the late secretion mode (Figure 4a): FliC is recognized, unfolded, and injected into the secretion channel by a late mode fT3SS, while the bulky, folded GFP remains at the pore entrance. Thereby the secretion of any other substrate would be blocked. However, HBBs that persisted in early secretion mode would not be affected and we hypothesized that under these conditions the observed secretion of early substrates in the late secretion mode strain would not be altered. If, however, the early substrates were indeed secreted by an fT3SS that had switched to late secretion mode, their secretion would also be abolished by the FliC-GFP plug.

As shown in Figure 4b,c, expression of the FliC-GFP plug substantially decreased, but did not entirely abolish, secretion of the late substrate FlgK. This could be explained by the fact that the plug also inhibits FlgM secretion from the cell, which subsequently prevents σ^{28} -dependent transcription of *flic-gfp* from its Class 3 promoter. Accordingly, we deleted the *flgM* gene ($\Delta flgM$) to ensure constitutive expression of the FliC-GFP plug. As anticipated, the cellular FliC-GFP levels strongly increased and FlgK secretion was almost

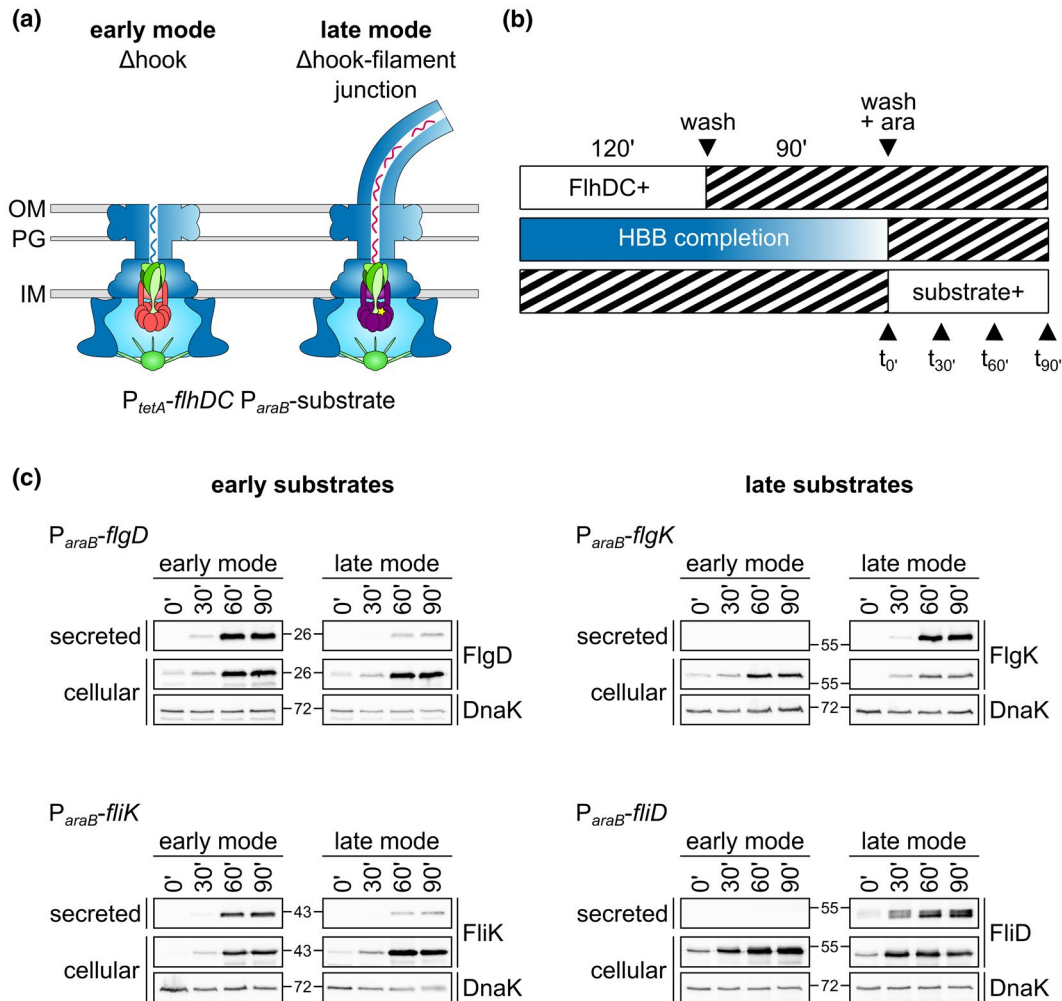


FIGURE 3 Specificity of early and late substrate secretion in FT3SSs operating in early or late secretion mode. (a) Schematic representation of the main features of the used secretion reporter strains. Strains were deleted for the hook (Δ *flgE*) or the hook-filament junction (Δ *flgKL*) proteins to obtain a population of cells locked in early or late mode of secretion, respectively. The flagellar master regulator *flhDC* was placed under an anhydrotetracycline-inducible promoter (P_{tetA} -*flhDC*) and individual early and late substrates were expressed from the chromosomally encoded P_{araB} promoter (P_{araB} -substrate). (b) Schematic of the experimental timeline indicating the time of FlhDC induction (FlhDC+), washing steps, the presumed time for hook-basal-body (HBB) completion and the induction of substrates from the P_{araB} promoter by addition of arabinose (ara). Samples were acquired 0, 30, 60, and 90 min after substrate induction. (c) Secreted and cellular levels of early (left) and late substrates (right) in early and late secretion mode strains. The induced substrate from the P_{araB} promoter is displayed on the top left-hand side of each representative Western blot. A protein size marker in kDa is indicated in the center of each Western blot. DnaK served as a cell lysis and loading control and was only detected in the cellular fraction. Individual proteins were detected using polyclonal (α -FlgD, α -FliK, α -FlgK, α -FliD) or monoclonal (α -DnaK) antibodies

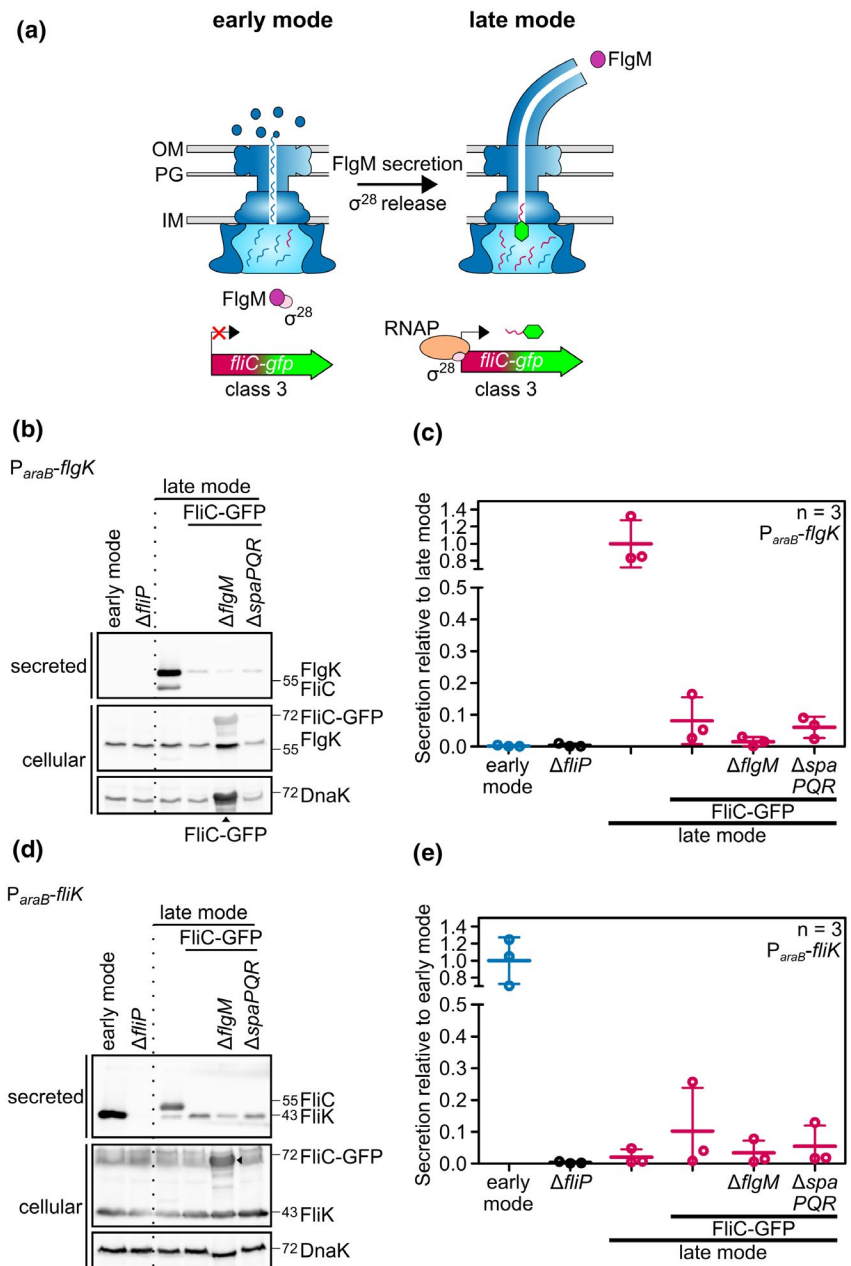
entirely abolished in a Δ *flgM* FliC-GFP background (Figure 4b,c). The remaining minimal secretion might result from the 14-fold higher binding affinity advantage of the FlgN-FlgK chaperone-substrate complex to FlhA in comparison to the FliS-FliC complex (Kinoshita et al., 2013) and possibly decreased binding efficiency of the FliS chaperone to the C-terminal FliC-GFP fusion protein. We furthermore excluded unspecific secretion via the injectisome T3SS or cell lysis by analyzing FlgK secretion in *fliP* (T3SS pore component) and *spaPQR* (VT3SS pore components) null mutants. Interestingly, and in contrast to the strongly reduced secretion of the late substrate FlgK, secretion of the early substrate FliK was not affected by expression of the FliC-GFP plug (Figure 4d,e), indicating that FliK was secreted by a fraction of HBBs that had not switched to late secretion mode

and that presumably remained in early secretion mode (Figure 2). These results demonstrate a remarkable specificity for early or late substrates of FT3SSs that are operating in either early or late secretion mode, respectively. Residual early substrate secretion in the late mode strain is likely due to a fraction of FT3SSs that remained in early secretion mode and the artificial increase of substrate availability by overexpression via the P_{araB} promoter.

3 | DISCUSSION

T3SSs are able to selectively secrete different classes of secretion substrates at different assembly stages of the macromolecular

FIGURE 4 *ft*3SSs operating in late mode specifically secrete late substrates. (a) Schematic representation of the mode of action of the FliC-GFP approach in order to plug *ft*3SSs operating in late secretion mode. (b and d) Secreted and cellular protein levels of the late substrate FlgK (b) and the early substrate FliK (d) in early and late mode strains expressing the FliC-GFP plug. (c and e) Quantification of three independent biological replicates of the exemplary secretion assays shown in panels b and d, respectively. A protein size marker in kDa is indicated on the right-hand side of each representative Western blot. DnaK served as a cell lysis and loading control and was only detected in the cellular fraction. Individual proteins were detected using polyclonal (α -FlgK, α -FliC, α -FliK) or monoclonal (α -DnaK) antibodies



machine. However, the mechanism of this remarkable substrate selectivity remains unclear. We investigated here in particular the question of substrate selectivity of the flagellar T3SS. We reasoned that two different mechanisms could account for the previously observed sequential secretion of (early then late) substrates via the *ft*3SS. Firstly, it appears possible that the substrate specificity switch results in conformational rearrangements within the export apparatus that change the substrate selectivity of the *ft*3SS completely from one substrate class to another: that is before the switch, the *ft*3SS recognizes only early substrates, and after the presumed conformational rearrangement (the switch), only late substrates are recognized and secreted. Such a complete switch in substrate selectivity would presumably be unidirectional and irreversible. A complete and irreversible switch would also facilitate the simultaneous assembly of multiple *ft*3SSs at different assembly stages and ensure

that substrates are only secreted at the appropriate stage of assembly. In this respect, recent results of Inoue et al. have suggested that the interaction of the flexible linker of FlhA (FlhA_L) with the cytoplasmic domain of FlhA (FlhA_C) might function as a structural switch that might contribute to the specificity switch of the *ft*3SS from hook-type to filament-type substrate secretion by suppressing the interaction of late substrate-chaperone complexes with FlhA_C before hook completion (Inoue et al., 2021).

Secondly, it also appears possible that conformational rearrangements within the export apparatus simply enlarge the substrate selectivity spectrum of the *ft*3SS so that it additionally recognizes and secretes a broader range of substrates: that is, after the switch, the *ft*3SS recognizes and secretes late substrates in addition to early substrates. Such a switch mechanism intuitively appears to be simpler mechanistically but might also waste more cellular resources as

early substrates would continue to be secreted, although no longer required for flagellar assembly post-switch.

Accordingly, in order to investigate these possibilities, we developed a synchronized secretion substrate reporter setup that allowed us to test secretion of the different substrate classes in fT3SSs operating in either early or late secretion mode (Figures 1 and 2). This approach differs from other studies which investigated late substrate secretion in a heterogeneous population (Hirano et al., 2003; Stafford et al., 2007).

Using the synchronized reporter setup, we found that fT3SSs operating in early mode were able to secrete only early-type substrates, whereas strains where the fT3SSs operated in late mode predominantly secreted late-type substrates (Figure 5). Surprisingly, we observed in the late-mode strains also a low level of early substrate secretion (Figure 3). Importantly, however, our initial experiments characterizing the synchronized reporter setup revealed that about 20% of the fT3SSs assembling a hook structure apparently did not switch to late secretion mode because these HBBs did not assemble a filament (Figure 2). Since we frequently observed individual cells, which displayed both filament-attached HBBs and HBBs without a filament, we concluded that not a general lack of late secretion substrate expression accounts for the defect in filament assembly. We speculate that during the HBB construction process a certain probability exists for assembly mistakes that render the fT3SS unable to undergo the conformational changes needed for the substrate specificity switch and thus complete hook assembly. This conclusion appears to be reasonable since the correct spatiotemporal assembly of complex, multicomponent macromolecular machines such as the flagellum is nontrivial. Hence, bacteria evolved sophisticated regulatory mechanisms in order to maximize the probability of a productive assembly, such as hierarchical gene expression of various substrates needed at different stages of assembly, different secretion substrate classes, as well as checkpoints coupling the assembly state to gene expression or substrate selectivity.

Accordingly, we conclude that the residual secretion of early substrates in the synchronized late mode strain is likely due to a fraction of fT3SSs that were unable to perform the switch to late secretion mode. Further evidence supports the conclusion that a minor fraction of fT3SSs remained in early secretion mode. We found that secretion of late-type substrates, but not early-type substrates, was prevented in the late mode strain by expressing a flagellin (late substrate)-GFP fusion protein, which specifically blocks secretion after the switch (Figure 4). This suggests that the switch to late mode secretion is irreversible since the residual early substrate secretion under late mode conditions was due to a minor fraction of fT3SSs that were unable to switch to late mode.

In summary, our results support a model of the substrate selectivity mechanism where the fT3SS undergoes a complete, unidirectional (i.e. from early- to late-type substrates) and irreversible (i.e. fT3SS in late secretion mode do not, or very infrequently, revert to early secretion mode) switch in substrate specificity (Figure 5). Such a mechanism, where after the switch, the fT3SS is unable to secrete early-type substrates would facilitate selection and secretion of only

those substrates required at a specific assembly stage under conditions where multiple flagella are assembled simultaneously but not synchronously within one cell.

Finally, the developed synchronized secretion substrate reporter strain provides a platform that will promote future investigations of the molecular mechanisms that underlie the elusive substrate recognition and substrate specificity switch of the fT3SS.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, and media

All bacterial strains used in this study are listed in Table 1. Cells were grown in lysogeny broth (LB) at 37°C. The generalized transducing phage of *Salmonella enterica* serovar Typhimurium P22 HT105/1 int-201 was used in all transductional crosses (Sanderson & Roth, 1988). Insertions and deletions were constructed using λ -RED homologous recombination (Datsenko & Wanner, 2000; Hoffmann et al., 2017). Media was supplemented with 0.2% L-arabinose (ara), 12.5 μ g/ml chloramphenicol, or 100 ng/ml anhydrotetracycline (AnTc) if appropriate.

4.2 | Synchronized secretion assay

An overnight culture of FliC phase-locked bacteria was inoculated 1:100 in LB supplemented with 100 ng/ml AnTc and incubated at 37°C, 180 rpm for 2 hr. Cells were washed with 1 \times PBS after centrifugation and resuspended in fresh LB after an additional centrifugation step. Incubation was pursued for 1.5 hr, and cells were washed again with 1 \times PBS as described before. The bacterial cell number was adjusted equally to OD₆₀₀ = 0.8 in all mutants according to the optical density measured at 600 nm (OD₆₀₀). The individual substrates from the P_{araB} locus were induced with 0.2% arabinose. A total amount of 1.9 ml culture was harvested at the indicated times. Cellular and supernatant fractions were collected by centrifugation (13,000 \times g, 3 min, 4°C). Proteins were precipitated by addition of 10% TCA and resuspended in 2 \times SDS sample buffer with 2% β -mercaptoethanol for subsequent protein analysis by SDS-PAGE and Western blotting. Proteins were detected using polyclonal (α -FliK, α -FliC, α -FlgD, α -FliD, α -FlgK) and monoclonal (α -DnaK) antibodies.

4.3 | Maleimide fluorophore labeling of the hook

In order to visualize the assembled hook structures, we used strains harboring a cysteine substitution within the hook protein FlgE (FlgE_{S171C}). The assay was performed as described for the synchronized secretion assay above, and the experimental timeline is presented in Figure 1 and Figure S2. Fifteen minutes before the first incubation step ended, 500 μ l culture was transferred into a microcentrifuge tube. Cells were pelleted at 2,500 \times g for 3 min and

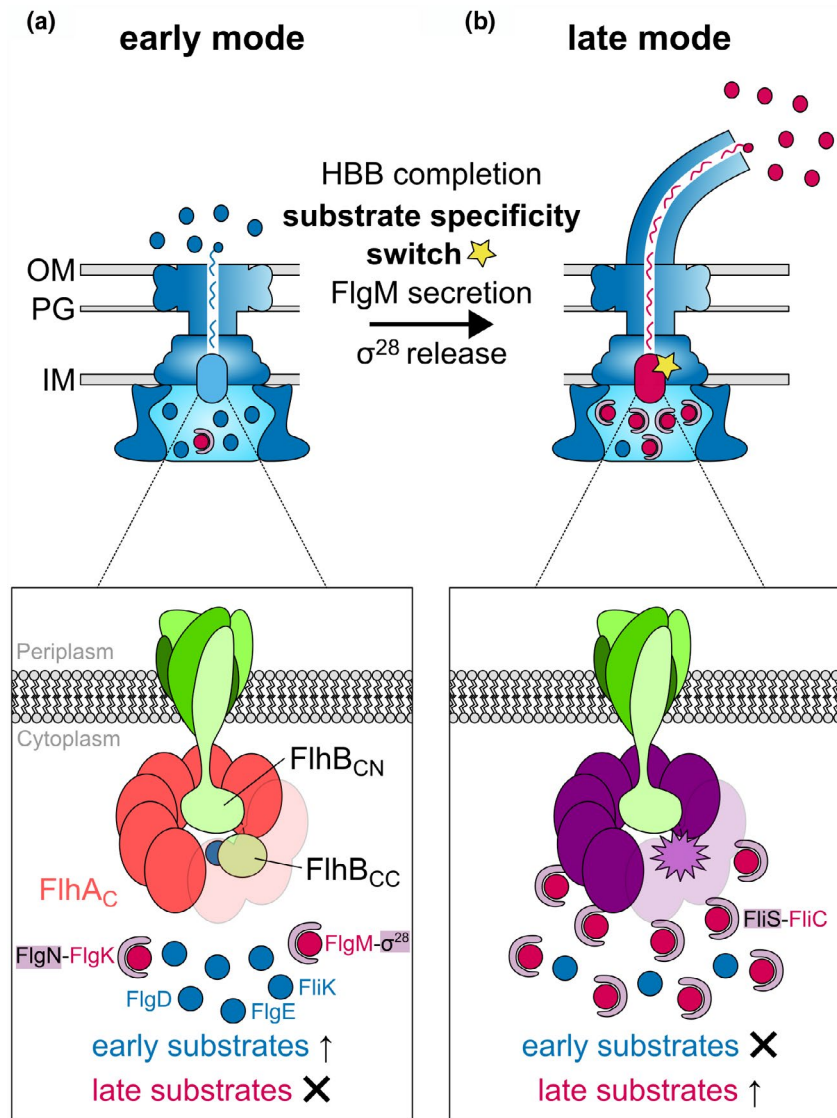


FIGURE 5 Schematic model of substrate selectivity of the fT3SS. The fT3SS features two distinct secretion modes during assembly: (a) early and (b) late mode. The ability to secrete substrates (↑) or not (✕) is indicated. (a) The early secretion mode is defined as the ability of the fT3SS to secrete early substrates (blue) before hook completion. The early substrates include, among others, the hook-basal-body (HBB) components and the molecular ruler FliK. Inlay panel: Schematic representation of the fT3SS displaying the FlhPQR-FlhB complex (shades of green) and the nonameric FlhA_C ring (orange). FlhB_C (light green) undergoes autocatalytic cleavage into the subdomains FlhB_{CN} and FlhB_{CC} before incorporation into the export apparatus. FlhB_{CC} provides binding sites for early substrates, while binding sites for late substrates on FlhA_C are occluded during HBB assembly. The early mode exclusively secretes early, but not late substrates. (b) Upon HBB completion, a switch in substrate specificity occurs (indicated by a yellow star), which initiates the late mode. Inlay panel: Conformational rearrangements of the substrate docking platform formed by FlhB_C and FlhA_C result in a highly specific and irreversible switch in substrate selectivity. The fT3SS switches from early to late substrate secretion by prohibiting early substrate recognition and concurrently providing binding sites for late substrates (magenta) in complex with their cognate chaperones (light magenta) on FlhA_C. Conformational rearrangements are indicated by a change in shape and color (FlhB_{CC}, light green to dark pink; FlhA_C, orange to dark purple). IM, inner membrane; OM, outer membrane; PG, peptidoglycan

resuspended in 1×PBS. All upcoming centrifugation steps were performed likewise if not indicated otherwise. A volume of 10 μg/ml DyLight™ 550 Maleimide (Thermo Fisher Scientific, 62290) was added and incubated for 10 min at 37°C, 300 rpm in a thermomixer. Samples were incubated under the same conditions in the upcoming steps if not indicated otherwise. Cells were pelleted and resuspended in 1×PBS. The wash step was repeated to reduce background fluorescence during image acquisition. A small proportion

of sample was transferred into a new tube and fixed with PFA at a final concentration of 2% for 10 min at the room temperature. After washing, the cells were applied to a homemade flow-cell and visualized at 100× magnification by epifluorescence microscopy (t_1). The remaining sample was centrifuged again, resuspended in fresh LB, and incubated for 75 min. The cells were pelleted, resuspended in 1×PBS, and 1 mM Maleimide-PEG₂-Biotin (Thermo Fisher Scientific, A39261) was added for masking newly formed

TABLE 1 List of *Salmonella enterica* serovar Typhimurium LT2 strains used in this study

Strain	Genotype	Reference
EM4872	$\Delta hin-5717::FRT (fliC^{ON}) flgE6506 (S171C) P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM9051	$\Delta hin-5717::FRT (fliC^{ON}) fliC6500 (T237C) flgE7742::3\times HA (after aa241) P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM7658	$\Delta araBAD978::flgK P_{flhDC5451}::Tn10dTc[del-25] \Delta flgE22964::FRT \Delta hin-5717::FRT (fliC^{ON})$	This study
EM7659	$\Delta araBAD978::flgK P_{flhDC5451}::Tn10dTc[del-25] \Delta flgKL5740::FRT \Delta hin-5717::FRT (fliC^{ON})$	This study
EM8757	$\Delta flgKL7770 \Delta hin-5717::FRT (fliC^{ON}) fliC5936 (FliC-GAGAGAGA-GFPmut2) \Delta araBAD978::flgK P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM8999	$\Delta flgKL7770 \Delta hin-5717::FRT (fliC^{ON}) fliC5936 (FliC-GAGAGAGA-GFPmut2) \Delta araBAD978::flgK \Delta spaPQR P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM8949	$\Delta flgKL7770 \Delta hin-5717::FRT (fliC^{ON}) fliC5936 (FliC-GAGAGAGA-GFPmut2) \Delta araBAD978::flgK P_{flhDC5451}::Tn10dTc[del-25] \Delta flgM5794::FRT$	This study
EM9483	$\Delta fliP6709 \Delta hin-5717::FRT (fliC^{ON}) \Delta araBAD978::flgK P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM7564	$\Delta araBAD7606::fliK P_{flhDC5451}::Tn10dTc[del-25] \Delta flgE22964::FRT \Delta hin-5717::FRT (fliC^{ON})$	This study
EM7565	$\Delta araBAD7606::fliK P_{flhDC5451}::Tn10dTc[del-25] \Delta flgKL5740::FRT \Delta hin-5717::FRT (fliC^{ON})$	This study
EM7568	$\Delta araBAD7606::fliK \Delta flgKL5740::FRT \Delta fliP6709(\Delta aa6-241) P_{flhDC5451}::Tn10dTc[del-25] \Delta hin-5717::FRT (fliC^{ON})$	This study
EM8758	$\Delta flgKL7770 \Delta hin-5717::FRT (fliC^{ON}) fliC5936 (FliC-GAGAGAGA-GFPmut2) \Delta araBAD7606::fliK P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM9000	$\Delta flgKL7770 \Delta hin-5717::FRT (fliC^{ON}) fliC5936 (FliC-GAGAGAGA-GFPmut2) \Delta araBAD7606::fliK \Delta spaPQR P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM8950	$\Delta flgKL7770 \Delta hin-5717::FRT (fliC^{ON}) fliC5936 (FliC-GAGAGAGA-GFPmut2) \Delta araBAD7606::fliK P_{flhDC5451}::Tn10dTc[del-25] \Delta flgM5794::FRT$	This study
EM5978	$\Delta araBAD980::fliD P_{flhDC5451}::Tn10dTc[del-25] \Delta flgE22964::FRT$	This study
EM5979	$\Delta araBAD980::fliD P_{flhDC5451}::Tn10dTc[del-25] \Delta flgKL5740::FRT$	This study
EM7566	$\Delta araBAD1217::flgD P_{flhDC5451}::Tn10dTc[del-25] \Delta flgE22964::FRT \Delta hin-5717::FRT$	This study
EM7567	$\Delta araBAD1217::flgD P_{flhDC5451}::Tn10dTc[del-25] \Delta flgKL5740::FRT \Delta hin-5717::FRT (fliC^{ON})$	This study
EM5502	$\Delta flgE1204 \Delta hin-5717::FRT (fliC^{ON}) P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM2046	$\Delta hin-5717::FRT (fliC^{ON}) fliC6500 (T237C) P_{flhDC5451}::Tn10dTc[del-25]$	This study
EM12185	$\Delta flgE1204 \Delta hin-5717::FRT (fliC^{ON}) P_{flhDC5451}::Tn10dTc[del-25] / pRG19::FCF (P_{motA}-luxCDABE, Cm^R, Tet^R)$	This study
EM11808	$\Delta hin-5717::FRT (fliC^{ON}) fliC6500 (T237C) P_{flhDC5451}::Tn10dTc[del-25] / pRG19::FCF (P_{motA}-luxCDABE, Cm^R, Tet^R)$	This study
EM12126	$\Delta hin-5717::FRT fliC6500(T237C) flgE7742::3\times HA (after aa241) P_{flhDC5451}::Tn10dTc[del-25] / pRG19::FCF (P_{motA}-luxCDABE, Cm^R, Tet^R)$	This study

HBBs. The cells were incubated for 10 min, subsequently pelleted again, and resuspended in 1×PBS. The wash step was repeated, and cells were resuspended in fresh LB. Incubation was pursued for 75 min. The cells were pelleted, resuspended in 1×PBS, and 10 µg/ml DyLight™ 488 Maleimide (Thermo Fisher Scientific, 46602) was added to label newly formed HBBs. The sample was incubated for 10 min and fixation as well as visualization was performed as described above (t_2).

4.4 | Simultaneous hook and filament labeling

For labeling the hook and filament structures simultaneously, we used a strain harboring a 3×HA epitope tag inserted into the hook protein ($FlgE_{3\times HA}$) and a previously described cysteine mutation on the filament subunits ($FliC_{T237C}$) for labeling with fluorophore-coupled maleimide dyes. The basic assay was performed as

described above for the synchronized secretion assay. For labeling of the hook and filament, 500 µl culture was transferred into a microcentrifuge tube after the last incubation step. The cells were pelleted at 2,500×g for 3 min and resuspended in 1×PBS. 10 µg/ml anti-HA-Alexa555 (Thermo Fisher Scientific, 26183-A555), and DyLight™ 488 Maleimide (Thermo Fisher Scientific, 46602) was added for sufficient labeling of both structures for 30 min with continuous shaking. Subsequently, the cells were washed twice with 1×PBS and fixed with PFA at a final concentration of 2% for 10 min at room temperature. After washing, the cells were applied to a homemade flow-cell and visualized at 100× magnification by epifluorescence microscopy. To exclude shearing of flagellar filaments due to harsh handling, samples were treated with different roughness as described before (Renault et al., 2017; Turner et al., 2012). Before labeling, the culture was passed 0, 10 (mild shearing), or 30 times (strong shearing) through a 22-gauge needle to cause mechanical shearing of flagellar filaments.

4.5 | Epifluorescence microscopy

Fluorescently labeled samples for simultaneous hook and filament labeling and hook multilabeling were visualized under a Zeiss Axio Observer Z1 inverted epifluorescence microscope. Z-stack images were acquired at 100× magnification with an Axiocam 506 mono CCD-camera using the Zen 2.6 pro software.

4.6 | Motility assay

Swimming motility assays were performed by inoculating 1 µl overnight culture in a 0.3% soft agar plate. The plate was incubated for 5 hr at 37°C, and the diameter of the swimming halo was measured using ImageJ. Values were normalized to the wild-type strain.

4.7 | Luciferase assay

Analyzed strains harbored the pRG19 plasmid encoding for a transcriptional fusion of the *luxCDABE* operon to the Class 3 promoter of *motA* (P_{motA} -*luxCDABE*) (Brown et al., 2008; Goodier & Ahmer, 2001), allowing to estimate the timing of switching from early to late secretion mode. An overnight culture was diluted 1:100 in 200 µl LB supplemented with 12.5 µg/ml chloramphenicol in a white walled 96-well plate. Luminescence and OD₆₀₀ were measured for 6 hr in 15 min intervals in a Synergy H1 microplate reader (BioTek).

ACKNOWLEDGMENTS

The authors thank Tohru Minamino for providing them with polyclonal antibodies (α-FlgD, α-FlgK, α-FlhD, α-FlhK) and Kelly T. Hughes for generous donation of strains. This work was supported in part by intramural funding of the Humboldt-Universität zu Berlin, the Deutsche Forschungsgemeinschaft (DFG) research grant no. ER 778/2-1, and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no. 864971). Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

AG and ME conceived and designed the study. AG, MH, and SH performed the experiments. AG, MH, SH, and ME analyzed the data. ME contributed reagents, materials, and funding. AG and ME wrote the paper. All authors edited the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Alina Guse  <https://orcid.org/0000-0002-2603-8571>

Manuel Halte  <https://orcid.org/0000-0001-9876-7493>

Svenja Hüsing  <https://orcid.org/0000-0003-1297-3312>

Marc Erhardt  <https://orcid.org/0000-0001-6292-619X>

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Guse, A., Halte, M., Hüsing, S. & Erhardt, M. (2021) Hook-basal-body assembly state dictates substrate specificity of the flagellar type-III secretion system. *Molecular Microbiology*, 116, 1189–1200. <https://doi.org/10.1111/mmi.14805>