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RESEARCH ARTICLE

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FlrA-independent production of flagellar proteins is required for proper flagellation in *Shewanella putrefaciens*

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Abstract

Flagella are multiprotein complexes whose assembly and positioning require complex spatiotemporal control. Flagellar assembly is thought to be controlled by several transcriptional tiers, which are mediated through various master regulators. Here, we revisited the regulation of flagellar genes in polarly flagellated gammaproteobacteria by the regulators FlrA, RpoN (σ^{54}) and FliA (σ^{28}) in *Shewanella putrefaciens* CN-32 at the transcript and protein level. We found that a number of regulatory and structural proteins were present in the absence of the main regulators, suggesting that initiation of flagella assembly and motor activation relies on the abundance control of only a few structural key components that are required for the formation of the MS- and C-ring and the flagellar type III secretion system. We identified FlrA-independent promoters driving expression of the regulators of flagellar number and positioning, FlhF and FlhG. Reduction of the gene expression levels from these promoters resulted in the emergence of hyperflagellation. This finding indicates that basal expression is required to adjust the flagellar counter in *Shewanella*. This is adding a deeper layer to the regulation of flagellar synthesis and assembly.

KEYWORDS
flagella, FleN, FleQ, FlhG, regulation

1 | INTRODUCTION

Many bacterial species are able to synthesize flagella, long proteinaceous helical fibres that extend from the cell's surface. Rotation of the flagellar helix, mediated by a membrane-embedded motor, enables efficient active movement of the cell through liquid environments (swimming) or across surfaces (swarming) (Kearns, 2010; Thormann et al., 2022; Wadhwa & Berg, 2022). In addition, the flagellum can serve as an adhesive or pathogenicity factor or as a sensor the bacteria use to determine environmental conditions such as viscosity or surface wetness (Chaban et al., 2015; Laventie & Jenal, 2020). The flagellum is an intricate nanomachine, which – in its general design – is well-conserved between different bacterial species. It consists of the helical filament, the cell envelope-embedded basal body housing the rotary motor and a type III export apparatus, and a universal joint structure, the hook, which connects the motor and filament (Figure 1a) (Johnson et al., 2021; Tan et al., 2021). The whole structure is formed from about 20 different protein building blocks with different stoichiometries ranging from one to nine copies, e.g. for parts of the flagellar type III export system (fT3SS), to many thousands of the main filament building block, the flagellin (Altegoer & Bange, 2015; Berg, 2003). The flagellar motor is rotated

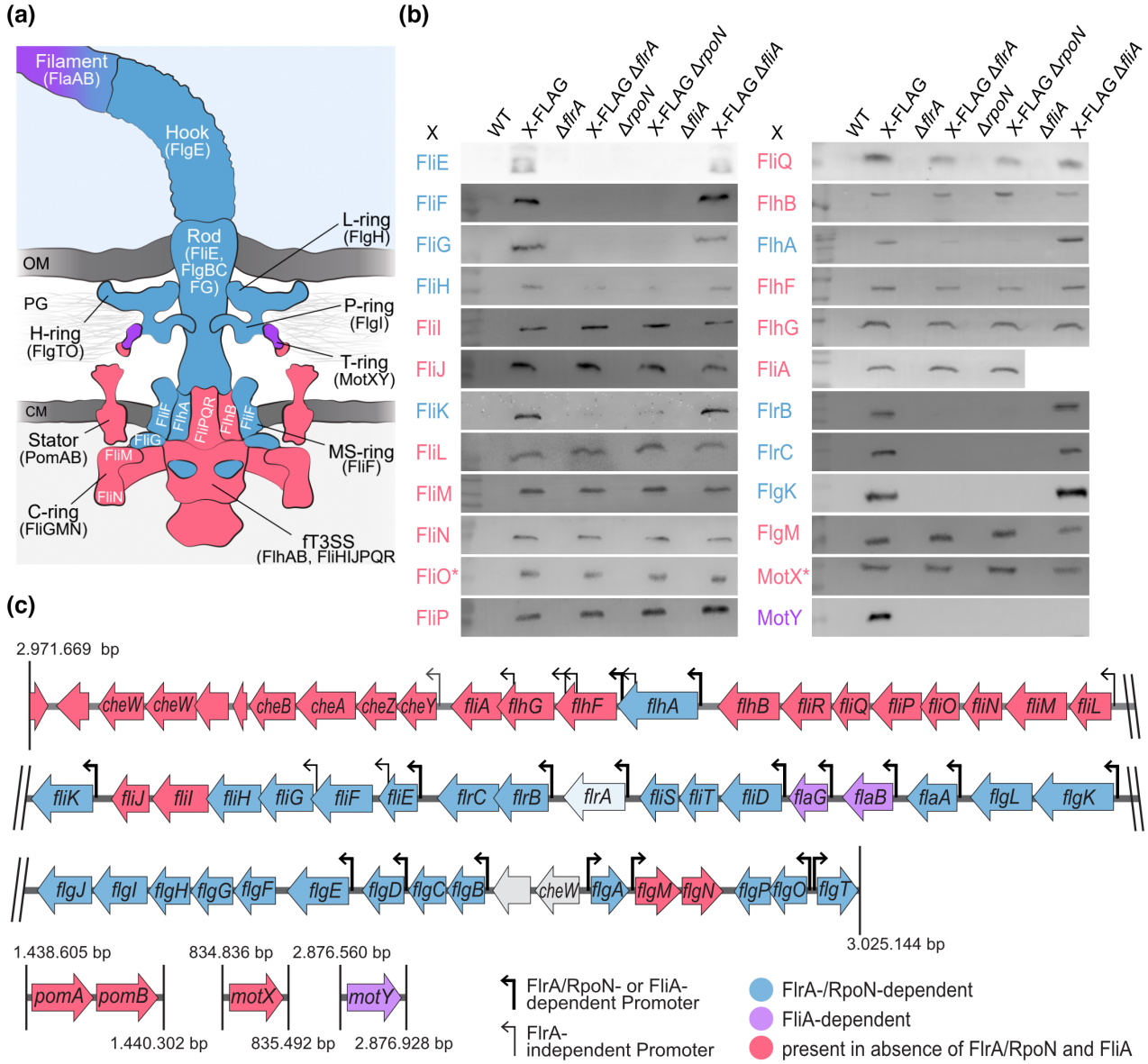

FIGURE 1 Presence of flagellar components in the absence of flagellar regulators. Flagellar components marked in red are also present in the deletion mutants of the regulators *flrA*, *rpoN* and *fliA*. Blue-labelled components are FlrA- as well as RpoN-dependent, and purple-labelled components are FliA-dependent. (a) Schematic representation of the flagellum. OM, outer membrane; PG, peptidoglycan, CM, cell membrane. (b) Western blot analyses of some flagellar components in deletion strains of the flagellar master regulator *flrA* or the sigma factors *rpoN* and *fliA*, respectively. The corresponding proteins were chromosomally fused with a 3xFLAG, sfGFP (FliO), or mCherry (MotX). The complete blots as well as Coomassie-stained SDS polyacrylamide gels serving as loading controls are shown in Figures S3 and S4. (c) Polar flagellar gene cluster and offside flagellar genes. The thick arrows represent the FlrA/RpoN- and FliA-dependent promoters known from *S. oneidensis*. Newly identified FlrA-independent promoters are shown with thin arrows. The numbers indicate the base position in the chromosome.

at the expense of transmembrane ion gradients, most motors either use H^+ or Na^+ as coupling ions (Sowa & Berry, 2008). As synthesis, assembly and operation require a considerable amount of energy and cellular resources, the formation and function of flagella are tightly regulated.

The regulation of flagellar synthesis has been extensively studied for some Gram-negative gammaproteobacteria. At the transcriptional level, a three-tiered regulation cascade has been well-established for *Salmonella enterica*, which leads to a stepwise production and assembly of the flagellar multiprotein complex (Chevance & Hughes, 2008). In polarly flagellated gammaproteobacteria, such as *Vibrio* sp. or *Pseudomonas aeruginosa*, a four-tiered transcriptional hierarchy underlies the expression, production and assembly of the flagellar apparatus (Dasgupta et al., 2003; Echazarreta & Klose, 2019). A variation of the four-tiered transcriptional cascade was shown to exist in the monopolarly flagellated gammaproteobacterium *Shewanella oneidensis* (Gao et al., 2018; Shi et al., 2014; Wu et al., 2011). In this and other *Shewanella* species, the vast majority

of flagella-related genes are clustered in several adjacent operons in the genome (Wu et al., 2011). Flagellar gene expression is initiated with the activation of the flagellar master regulator FlrA. In concert with sigma factor 54 (σ^{54}; RpoN), FlrA activates the expression of a number of genes encoding most building blocks of the cell envelope-embedded basal body and the hook. In addition, FlrA/σ^{54} induces the expression of several other regulators, amongst which is the two-component system FlrBC. In polar flagellates such as *Campylobacter* (here designated FlgSR), *Vibrio* or *Pseudomonas* (here designated FleSR), these two-component regulators are thought to form a checkpoint that monitors MS-ring, C-ring and fT3SS assembly to then activate the genes encoding the building blocks for the flagellar rod and hook (Boll & Hendrixson, 2013; Burnham et al., 2020; Dasgupta et al., 2003; Echazarreta & Klose, 2019; Joslin & Hendrixson, 2009). In contrast, in *S. oneidensis* these genes are under the direct control of FlrA/σ^{54}. In *Shewanella*, FlrBC appears to be generally dispensable for flagella synthesis, but may be involved in regulating the composition, mechanical properties and behaviour of the flagellar filament (Gao et al., 2018; Kühn et al., 2017, 2018; Shi et al., 2014).

Amongst the other regulators thought to be under the direct control of FlrA is the sigma factor σ^{28} (FliA), which is responsible for expression of the late flagella factors, i.e., some motor components and the main flagellin. FliA is bound and thereby inactivated by its anti-sigma factor FlgM to prevent the production of large amounts of flagellin before the assembly of the filament can start. Only upon completion of the hook structure, FlgM is excreted from the cell and the released σ^{28} initiates the expression of its corresponding promoters (Chevance & Hughes, 2008).

In polarly flagellated gammaproteobacteria, the flagella synthesis synthesis needs to be spatiotemporally regulated to target assembly to the designated cell pole. In addition, the cells use a counting mechanism that restricts the number of flagella. In many bacterial species, proper targeting and counting are mediated by two proteins, FlhF and FlhG, which, in *Shewanella*, are proposed to reside in the second tier of the transcriptional hierarchy under the control of FlrA (Gao et al., 2018; Shi et al., 2014; Wu et al., 2011). In the absence of FlhF, an SRP-type GTPase, fewer cells are flagellated and the flagellum is frequently detached from the cell pole (Blagotinsek et al., 2020; Gao et al., 2015; Rossmann et al., 2015; Schuhmacher, Rossmann, et al., 2015a). The MinD-like ATPase FlhG antagonizes the function of FlhF at the cell pole and plays a major role in the counting mechanism that restricts the number of polar flagella to one (Blagotinsek et al., 2020; Gao et al., 2015; Schuhmacher, Rossmann, et al., 2015a; Schuhmacher, Thormann, & Bange, 2015b). Our previous work indicated that, in *S. putrefaciens*, an FlhG monomer binds to the C-ring building block FliM and passively travels to the nascent flagellar basal body. Assembly of FliM into the cytoplasmic C-ring (see Figure 1a) leads to the release of FlhG, which then binds ATP to form a dimer. The ATP-bound FlhG dimer, in turn, directly interacts with the main flagellar regulator FlrA, which prevents the transcription of genes encoding further early basal body building blocks. Whilst there are still several open questions, the current working model suggests that free ATP-bound FlhG dimers antagonize FlrA-mediated transcription of key flagellar genes once the C-ring (and the flagellar basal body) are complete. By this, FlhG connects flagellar assembly with transcriptional control of the corresponding building blocks and prevents the formation of additional flagella (Blagotinsek et al., 2020; Schuhmacher, Rossmann, et al., 2015a). A similar spationumerical regulation mechanism has been shown to be active in *P. aeruginosa* with the FlhG and FlrA orthologs FleN and FleQ (Chanchal et al., 2021). To function properly, one important parameter of the model is a certain equilibrium between the copy numbers of the involved structural and regulatory proteins.

During our investigations on the interplay between FlhG and FlrA, we noticed that in mutants in which *flrA* was deleted, FlhG was present at levels similar to those of wild-type cells (Blagotinsek et al., 2020), which did not concur with the current models stating that FlhG expression and production is under control of FlrA (Gao et al., 2018; Shi et al., 2014; Wu et al., 2011). This prompted us to revisit the current flagella regulation model by determination of mRNA and protein levels in *S. putrefaciens* wild-type cells and mutants lacking the main flagellar regulators FlrA, RpoN (σ^{54}) or FliA (σ^{28}). We found that the level of a number of early basal body proteins and the regulators FlhF, FlhG and FliA remains unchanged in the absence of FlrA or RpoN. Accordingly, we identified several promoters, which are independent of the known flagellar regulators that are driving the transcription of genes encoding these components. Lowering the activity of the newly identified promoters driving expression of *flhF*, *flhG* and *fliA* disturbed the numerical control of flagella synthesis, likely via FlhG. The data support a model in which constant production of flagellar components (e.g. FlhG) is required in *Shewanella* to maintain the regulatory circuit that prevents the formation of too many flagella, which may similarly occur in other monopolarly flagellated bacteria.

2 | RESULTS

2.1 | Determining the regulons of RpoN, FlrA and FliA in *S. putrefaciens*

The presence of FlhG in the absence of FlrA (Blagotinsek et al., 2020) lead us to speculate that the flagellar master regulator FlrA may not or only partially regulate the expression of flagellar genes and the presence of the corresponding encoded proteins. In addition, little data exists on the regulation of flagellar genes by FlrA in *Shewanella*, and, so far, the presence and position of most potential FlrA-dependent promoters rely on in silico predictions in *S. oneidensis*. As in the latter species, most genes required for the formation of the main polar flagellum of *S. putrefaciens* are clustered in a single genomic region, and the gene organization closely resembles that of *S. oneidensis* (Bubendorfer et al., 2012; Wu et al., 2011). Other genes encoding proteins directly related to flagellar synthesis and function, the stator proteins PomA and PomB, the T-ring proteins MotX and MotY, and the motor switch-effector ZomB, are scattered

throughout the chromosome (Brenzinger et al., 2018; Bubendorfer et al., 2012). In addition, *S. putrefaciens* harbours a secondary flagellar system, whose genes are clustered as a single operon elsewhere on the chromosome and which has no known role in the regulation of the primary polar system (Bubendorfer et al., 2012).

To generate a comprehensive data set on the role of the main flagellar regulators in *S. putrefaciens*, we determined both mRNA and protein levels in mutant strains in which *flrA* (Δ*flrA*), *rpoN* (Δ*rpoN*) or *fliA* (Δ*fliA*) were removed from the genome by *in-frame* deletion. Earlier studies indicated that loss of the two-component system FlrBC, which in other species, such as *Vibrio cholerae*, is crucial for flagella formation (Shi et al., 2014), has no effect on flagella synthesis in *S. putrefaciens* under our standard experimental conditions (Kühn et al., 2018). To confirm this, flagella-mediated swimming of Δ*flrBC* mutants was determined and compared to that of wild-type cells and mutants bearing mutations in other main transcriptional regulators (Δ*flrA*; Δ*fliA*; Δ*flrABC*). Whilst in the latter mutants spreading in soft agar was greatly diminished or completely abolished as expected, the Δ*flrBC* mutant showed no difference to wild-type spreading (Figure S1a). Therefore, we concluded that the FlrBC two-component system has a similar role as in *S. oneidensis* and hence did not further consider *flrBC* in this approach.

RNA and protein levels of Δ*flrA*-, Δ*rpoN* and Δ*fliA*-mutant cells were determined during the early exponential growth phase and compared to those of wild-type cells cultivated under the same conditions. As expected, the largest change occurred in the Δ*rpoN* mutant compared to the WT with 263 higher and 285 lower gene transcript levels. 104 genes were differentially regulated in the Δ*flrA* mutant amongst which 50 had higher and 54 had lower RNA levels (Table S1). In the absence of *fliA*, 102 genes were expressed at higher and 80 genes at lower levels (Table S1). As expected, many of the corresponding gene products have documented roles in flagella synthesis, but also a number of different genes not apparently related to flagella-mediated motility are directly or indirectly regulated by RpoN, FlrA and FliA at the transcriptional level.

At the protein level, FlrA-, RpoN or FliA-dependent changes were much less pronounced. The mass spectrometry approach identified 71 proteins in Δ*rpoN* mutants with significantly different abundances, 43 proteins in Δ*flrA* mutants, and 35 proteins in Δ*fliA* mutants compared to the wild-type proteome (Table S2). Changes at the protein levels were reflected at the transcriptome levels, as genes encoding proteins that exhibited higher or lower abundance were similarly up or downregulated in the corresponding mutants. For further analysis, we concentrated on those gene products that were directly or indirectly affected by RpoN, FlrA or FliA at the level of both RNA and protein (see Figure S2).

For FlrA, the vast majority of these gene products were either directly associated with the primary flagellar system or the Bpf surface adhesion system, which has previously been found to be regulated by FlrA in *S. putrefaciens* (Cheng et al., 2017). An exception was the genes encoding the flagellins specific for the secondary lateral flagella (*flaA*$_2$ and *flaB*$_2$, as opposed to the primary flagellin genes *flaA*$_1$ and *flaB*$_2$), indicating some FlrA-mediated regulatory cross-talk between the two flagellar systems. Besides the flagellar components and the Bpf surface adhesion system, some other genes and gene products appear to be regulated by RpoN, FlrA and FliA (see Tables S1 and S2). These apparent flagella-unrelated genes and proteins were not considered further in this study.

2.2 | Several flagellar proteins are present in the absence of FlrA, RpoN and FliA

The combined transcriptome and proteome analysis generally confirmed the expected role of FlrA as a major flagellar regulator. However, expression and protein levels of several flagellar genes and the corresponding gene products did not exhibit major differences in the absence of *flrA*. As some flagellar proteins were not detected at sufficient reliability in the global mass spectrometry approach, the results were validated by western immunoblotting. To enable specific detection of the appropriate proteins, strains were constructed in which the corresponding genes were extended on the chromosome by a sequence adding a C- or N-terminal 3xFLAG-tag as a suitable epitope to the produced protein. This way, we labelled FliE, FliF, FliG, FliH, FliI, FliJ, FliK, FliL, FliM, FliN, FliP, FliQ, FlhA, FlhB, FlhF, FlhG, FliA, FlrB, FlrC, FlgK, FlgM and MotY. For the detection of FliO, the protein was fused C-terminally to sfGFP and for MotX a suitable N-terminal mCherry fusion was already established. Most labelled proteins were stably produced and supported flagella-mediated motility to almost wild-type levels (Figures S1b, S3 and S4). Exceptions were the FLAG-tagged versions of FliE, FliK, FliN, FliQ and FlgK, which did not lead to functional flagella synthesis and the FliG- and FliP-FLAG mutants that were negatively affected in spreading through soft agar. Stable production still allowed to determine general differences in the presence of these proteins. To determine the presence or absence of the proteins in the presence of FlrA, RpoN or FliA, the tagged variants were introduced into *S. putrefaciens* strains lacking *flrA*, *rpoN* or *fliA* (Figure 1b).

Taken together, the global transcriptional and proteome data and immunoblotting approach indicate that several genes are transcribed and the corresponding proteins are produced in the absence of FlrA or FliA (summarized in Figure 1a,c). These comprise a whole gene cluster, *fliLMNOPQRflhB*, which likely forms an operon and encodes proteins required for the formation of part of the cytoplasmic C-ring and the ft3SS. Other early flagellar proteins produced in the absence of FlrA are further components of the ft3SS, FliI and FliJ. Also, FlrA-independent were the regulators FlhF, FlhG (as previously identified) and the σ^{28} factor FliA, which are thought to constitute a transcriptional unit. Similarly present is the anti-sigma factor for σ^{28}, FlgM, along with the cytoplasmic export chaperone FlgN. Also here, both corresponding genes likely form an operon. Surprisingly, the protein levels of motor components PomA and PomB, the flagellar stators and the T-ring protein MotX, which is required for stable recruitment of the stators into the flagellar motor, also remained constant in the absence of FlrA and RpoN. Almost all other components showed a pronounced downregulation at the transcriptional level

upon deletion of *flrA* or *rpoN*, and the corresponding proteins could not be detected. Amongst these genes and gene products are some components required for initiation of basal body formation, e.g., the ft3SS component FlhA, the MS- and C-ring proteins FliF and FliG, and virtually all components of the rod, the outer rings, the hook and the flagellins. As previously predicted, σ^{28} (FliA) was found to be crucial for the expression and production of the main flagellin $FlaB_1$, FlaG, and, in addition, also for the lacking T-ring component MotY.

2.3 | Identification of FlrA-independent promoters driving expression of flagellar genes

Our results demonstrated that the presence of a set of flagellar proteins is not dependent on FlrA. This indicated that the corresponding gene expression may occur even when *flrA* is deleted. We, therefore, determined if additional FlrA-independent promoters may drive the expression and production of at least some flagellar genes and proteins. To this end, a 400 to 550 bp DNA region upstream of selected flagellar genes, which putatively contained such FlrA-independent promoter regions, were amplified and fused to the *Photorhabdus luminescence luxCDABE* operon on a broad-host-range plasmid. Based on the occurrence of proteins and the predicted operon structure (Figure 1c), we selected regions upstream of the following genes: *fliF*, *fliG*, *fliH*, *fliI*, *fliK*, *fliL*, *flhA*, *flhF*, *flhG* and *fliA*. The region upstream of *fliE*, bearing a highly conserved FlrA-binding site, was used as a positive control. The plasmids were transformed into *S. putrefaciens* wild-type, Δ*flrA* and Δ*flrABC* cells, and emission of light due to the expression of the *lux* genes was measured in the presence and absence of FlrA (Figure 2a). As expected, the positive control bearing the upstream region of *fliE* displayed a strict FlrA-dependent expression. A similar pattern occurred for *fliK* and *flhA*, suggesting that the expression of the two genes is predominantly or exclusively governed by FlrA. In sharp contrast, for the *fliF*, *fliG*, *fliL*, *flhF*, *flhG* upstream regions *lux* expression occurred both in the presence and absence of FlrA. According to the determined light emission, the highest promoter activity is located in front of *fliL*. Also, the *fliA* upstream region enabled *lux* expression, albeit at a lower level. For *fliF*, *fliG*, *flhG* and *fliA*, there was no significant difference in expression in both the wild-type and Δ*flrA* strains. Expression initiated from the upstream regions of *fliL* and *flhF* was lower in the absence of FlrA, however, still robust luminescence occurred. These results strongly suggested that within these regions, FlrA-independent promoter regions are present that drive expression of the downstream regions. No promoter activities were detected for the *fliH* and *fliI* genes (Figure S5).

To identify the corresponding promoter region, the promoter-containing DNA fragments were truncated until loss of expression occurred to roughly home in on the position of the promoters (Figure S5). Then, we performed Rapid Amplification of 5' cDNA-Ends (RACE) PCR experiments using the *lux*-based reporter plasmids as templates in *S. putrefaciens*. By this, several putative transcriptional start sites were identified and mapped upstream of *fliF* (160 bp; $fliF_{-160}$), $fliG_{-86}$, $fliK_{-77}$, $fliL_{-91}$ and $fliA_{-249}$ (Figure 2b). Two potential transcriptional start sites occurred upstream of *flhF* ($flhF_{-403}$; $flhF_{-255}$) and flhG ($flhG_{-486}$; $flhG_{-297}$). Based on consensus sequence predictions in *Shewanella* (Shao et al., 2014), the deduced −10 and −35 regions of $fliF_{-160}$, $fliG_{-86}$, $fliL_{-91}$, $flhF_{-403}$, and both *flhG* putative transcriptional start sites ($flhG_{-486}$ and $flhG_{-297}$) may represent (degenerated) σ^{70}-dependent promoters. Homologies of the deduced −10 and −24 regions upstream of $fliA_{-249}$, $fliK_{-77}$ and $flhF_{-255}$ to the binding sequence of RpoN (σ^{54}) indicated that these potential promoters may be driven by RpoN, the main sigma factor involved in flagella synthesis. For an overview of the newly mapped promoters' position, see Figure 1c.

2.4 | FlrA-independent production of FlhG is required for proper numerical control of flagella synthesis

The presence of a number of constitutively active promoters within the operons encoding part of the flagellar basal body as well as the spatial and numerical regulators FlhG and FlhF and the sigma factor enabling the production of the main flagellin $FlaB_1$, FliA, suggested that constant protein production may have a role in flagellar synthesis. This particularly applied to FlhF and FlhG as changes in the levels of these proteins affect the number of polar flagella in *S. putrefaciens* (Blagotinsek et al., 2020; Schuhmacher, Rossmann, et al., 2015a). To determine a potential effect on flagellar synthesis, we aimed at silencing the promoters on the chromosome in the wild-type background. This was complicated by the fact that the identified promoters exhibit poor consensus sequence conservation and were, additionally, often located within the upstream structural gene of the same operon. We, therefore, introduced base substitutions within the predicted operons sequences without changing the encoded protein's codons or disturbing the open reading frame of the corresponding gene (Table S3). The effect of the base substitutions on transcriptional activity was first determined using the *lux*-based reporter systems (Figure 3a). By this, we were unable to generate substitution mutants with a notable effect for the promoter upstream of *fliL*. In contrast, we successfully silenced the promoter upstream of *flhF* within the gene *flhA* (P1; Figure 3a), another promoter upstream of the two promoters within *flhF* (P2) and diminish the activity of the third, more downstream (P3), both in front of *flhG*. These mutations were then introduced into their native positions within the chromosome, and the protein levels of FlhF and FlhG were determined by western blotting (Figure 3b). Both proteins were absent in strains bearing silenced promoters and lacking *flrA*, but produced to some extent in the presence of either *flrA* or the internal promoters. This was indicating that both proteins are controlled by the master regulator FlrA but are also produced by the *flrA*-independent promoters. The strain with the three substituted promoters was then tested on its ability to assemble flagella and spread through soft agar. Despite the production and presence of residual FlhG levels, the mutant exhibited a significant phenotype: whilst the number of flagellated cells

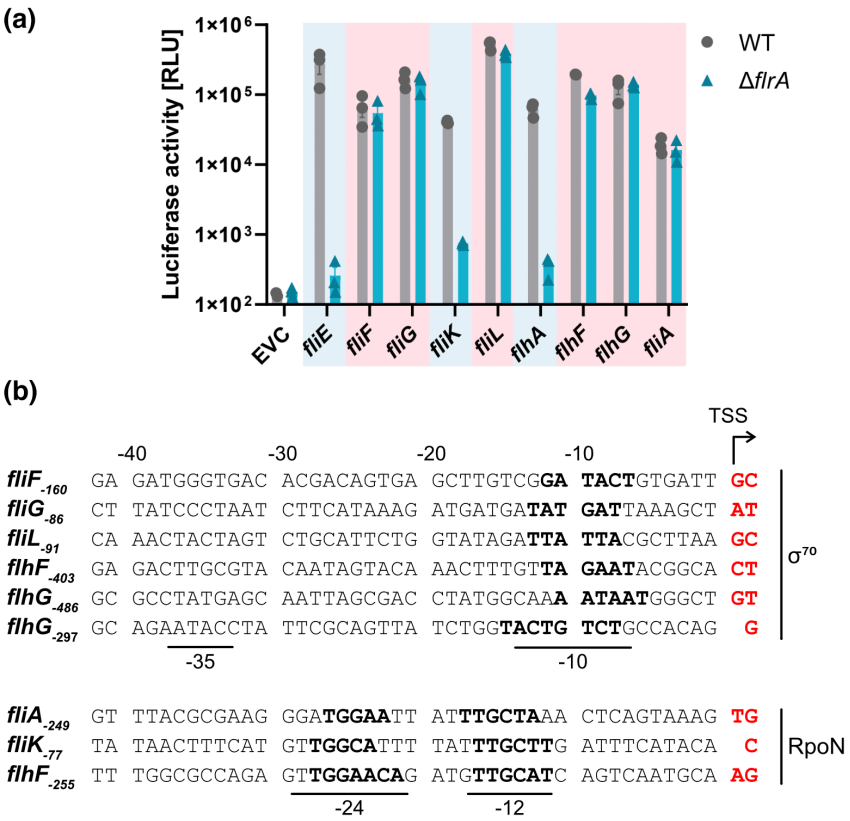

FIGURE 2 Some promoters of early flagellar components are not dependent on FlrA. (a) Determination of the promoters' transcriptional activity using a luciferase reporter system. The 400 or 550 bp fragments upstream of the translational start were fused to the reporter genes *luxCDABE* from *Photorhabdus luminescence*. These reporter plasmids with the promoter regions of interest were analyzed in *S. putrefaciens* WT and a mutant lacking the master regulator *flrA* (Δ*flrA*). The promoters highlighted in blue are FlrA-dependent and those highlighted in red are active in the absence of FlrA. Shown are the means and standard deviations from biological and technical triplicates. (b) Identification of transcription start sites (TSS) by 5' RACE-PCR. Bases highlighted in red and the curved arrow indicate the TSS. In the DNA sequence upstream from the TSS, potential binding motifs of the transcription factors of σ^{70} (−10 and − 35 region) and RpoN (−12 and − 24 region) were highlighted in bold. The numbers at the genes indicate the base pairs for the distance from the TSS to the translational start.

was highly similar in wild-type and mutant cells (about 75%), about 25% of the mutants cells synthesized more than one filament at the cell pole, which was never observed for the wildtype (Figure 3c,d). Also, the spreading capability of the mutant cells through soft agar was significantly decreased (Figure 3e).

Taken together, the results strongly indicate that expression from *flrA*-independent promoters and production of FlhG and maybe FlhF is required to maintain its role as a numerical regulator of flagellar synthesis.

3 | DISCUSSION

In this study, we have used complementary transcriptomic and proteomic approaches to investigate the regulatory network underlying the synthesis of the single polar flagellum in *S. putrefaciens*. Particularly, the quantification of protein levels allowed us to identify flagellar components and regulators whose production is strictly controlled. Jointly, the results demonstrate that the *S. putrefaciens* flagellar regulation significantly deviates from the four-tiered regulatory pathway model that is commonly proposed for monopolarly flagellated species such as *Vibrio* or *Pseudomonas* (Bouteiller et al., 2021; Dasgupta et al., 2003; Echazarreta & Klose, 2019). We show that, under standard conditions used in the experiments, a number of flagellar proteins including those forming the chemotaxis systems are produced in the absence of FlrA (see Figure 1c), and that production of just three proteins directly related to flagellar synthesis, FlaB, FlaG and MotY, is controlled by FliA (σ^{28}). All residual flagellar proteins depend on FlrA/RpoN, whilst the regulators FlrB and FlrC are not required. Taken together, the data indicate that, at least under these conditions, in *S. putrefaciens* a two-tiered flagellar transcriptional regulatory pathway is sufficient to efficiently synthesize a polar flagellum (Figure 4). We propose that the flagellar master regulator FlrA (in concert with σ^{54} [RpoN]) activates the expression and production of numerous flagellar building blocks, which, together with the already present FlrA-independently produced components result in the localized assembly of the flagellar basal body, the rod and the hook. Completion of the hook then allows the export of the anti-sigma factor FlgM to release σ^{28} (FliA) (Chevance & Hughes, 2008). Free σ^{28} in turn enables the initiation of synthesis

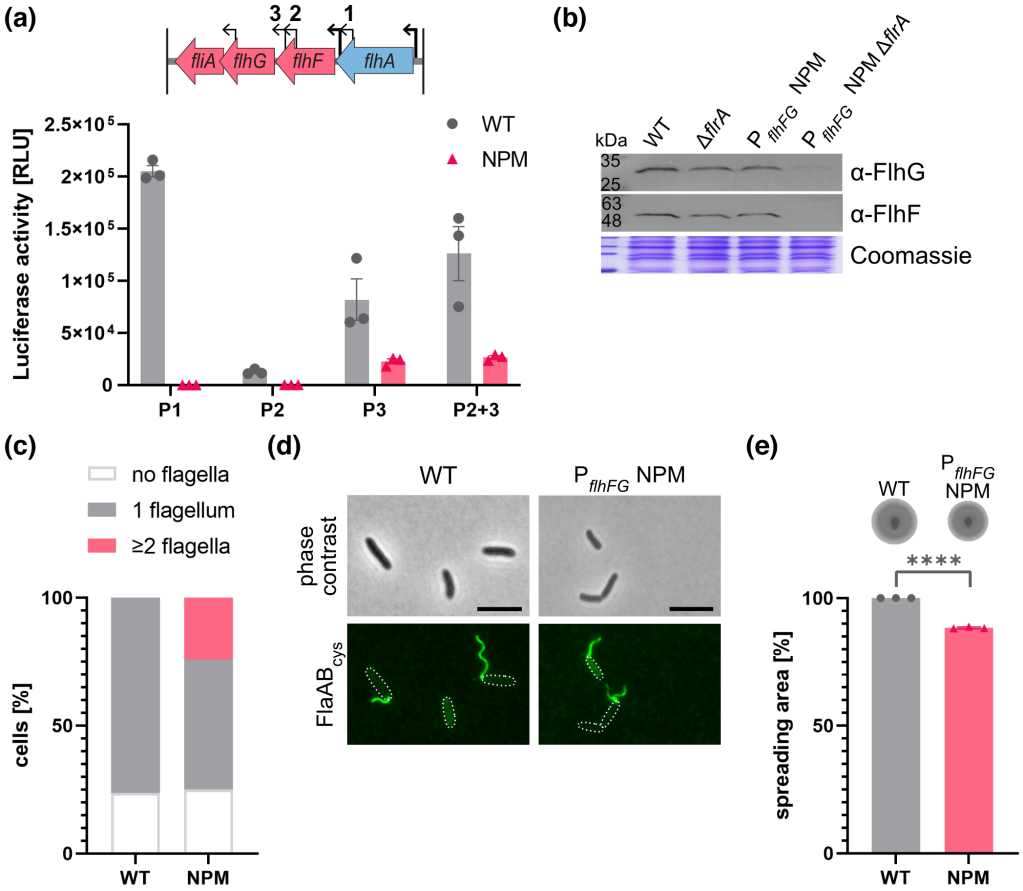

FIGURE 3 Constitutive production of the flagellation regulators FlhF and FlhG are essential for monopolar flagellation. (a) Top, overview of the *flhA* operon is shown with the regulation of gene expression (blue: FlrA/RpoN-dependent, red: present in Δ*flrA*, Δ*rpoN*, Δ*fliA*) and all promoters (thick arrow: FlrA/RpoN-dependent promoter, thin arrow: FlrA-independent promoter). Below are the measurements of promoter activities using the luciferase reporter system. The codon usage of the sequences of the FlrA-independent promoters of *flhF* (P1) and *flhG* (P2, P3, P2 + 3) was substituted (NPM, no promoter motif) to obtain the lowest possible promoter activity and compared with the native promoter sequence (WT). Shown are the means and standard deviations from biological and technical triplicates. (b) Western blot to check the protein levels of FlhF and FlhG in backgrounds of *flrA* deletion (Δ*flrA*), low promoter activity of *flhFG* (P_{flhFG} NPM) genes, and the P_{flhFG} NPM Δ*flrA* combination. Coomassie-stained SDS-polyacrylamide gel serves as a loading control. The complete blots are shown in Figure S4d. (c) Quantification of the number of flagella per cell. Flagellins were labelled via cysteine exchange using the fluorescent dye maleimide-CF484. Quantification was performed using 400 cells in biological triplicates. (d) Microscopy images of flagellar staining of the P_{flhFG} NPM strain compared with the WT. The scale represents 5 μm. (e) Top, the spreading of the *S. putrefaciens* WT and chromosomal mutant NPM (P_{flhFG} NPM) showing the lowest possible promoter activity of all three FlrA-independent promoters of *flhFG* are shown on soft-agar plates. Below is the per cent spreading radius from these two strains quantified. The per cent spreading radius of P_{flhFG} NPM was normalized to the WT. The experiment was performed in biological triplicates. Asterisks indicate that the difference from the WT is significant with a *p*-value of <0.0001 (two-sample t-test).

of the major flagellin FlaB and the T-ring component MotY. Whilst FlaB is being produced, the already synthesized minor flagellin FlaA can be exported and assembled to form the cell proximal part of the flagellar filament. This results in the typical spatial distribution of flagellins in the *S. putrefaciens* filament that optimizes its stability for a range of environmental conditions (Kühn et al., 2018). Together with MotX, MotY assembles the periplasmic T-ring, which allows functional coupling of the already produced PomAB stators into the now completed flagellar motor, which can start the rotational movement of the still-growing flagellum. The function of the third σ^{28}-controlled flagellar protein FlaG is yet elusive. Based on the findings in other species, FlaG may have a role in regulating the length of the filament (Capdevila et al., 2004; Inoue et al., 2018; McGee et al., 1996). This updated regulation model of *Shewanella* flagellation indicates that flagellar assembly in gammaproteobacteria can generally occur also in the absence of extensive transcriptional regulation, similar to that of the highly homologous type III secretion system (Diepold & Armitage, 2015; Diepold & Wagner, 2014).

A notable feature of the less intricate flagellar transcriptional hierarchy in *S. putrefaciens* is the missing requirement of the two-component system FlrBC, which has previously been demonstrated also for the closely related species *S. oneidensis* (Gao et al., 2018; Shi et al., 2014). In *V. cholerae* and *P. aeruginosa*, the corresponding orthologs (FlrBC in *Vibrio* and FleSR in *Pseudomonas*) are crucial for

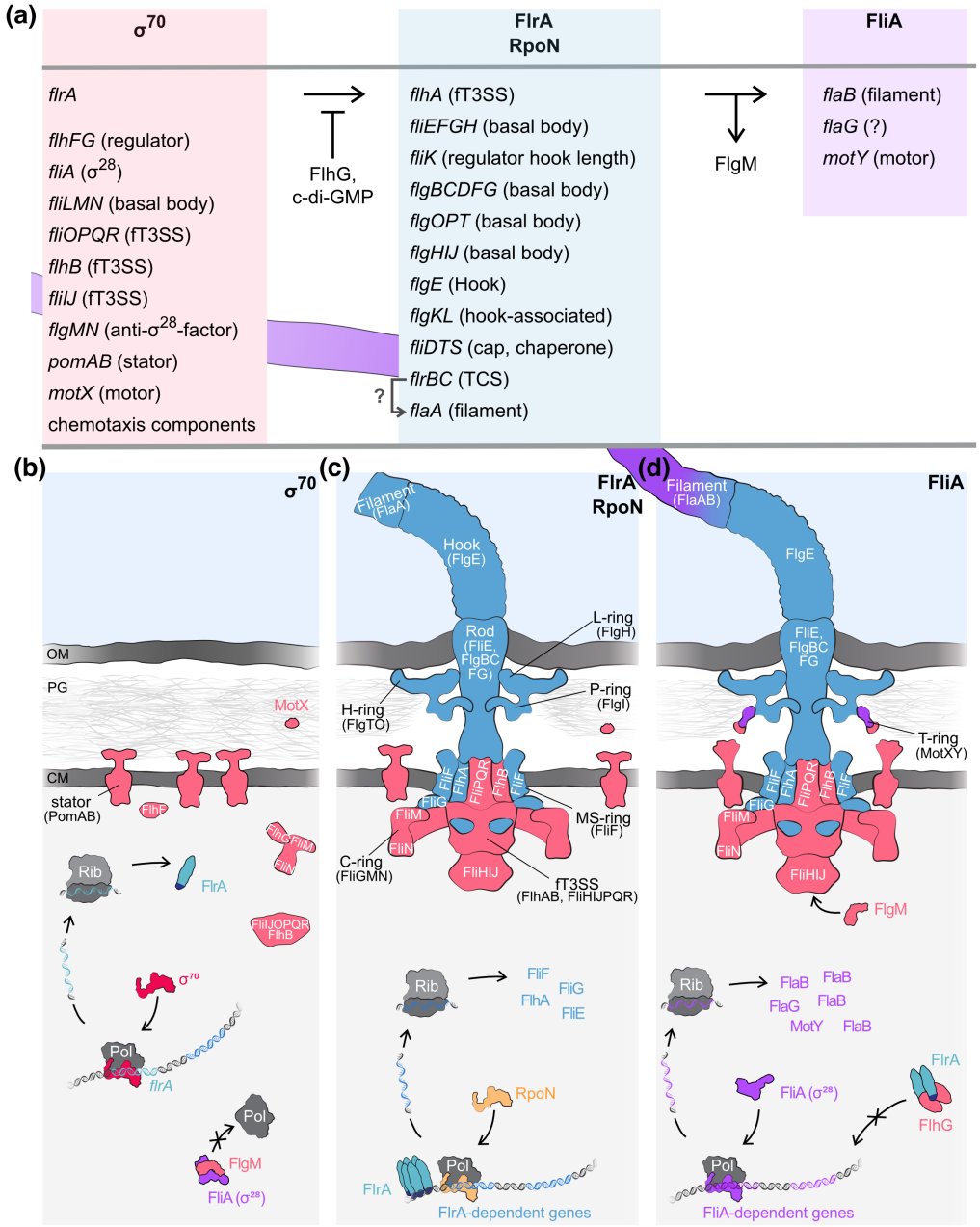

FIGURE 4 Transcriptional regulation model of polar flagellar synthesis in *S. putrefaciens*. (a) Transcriptional hierarchical regulation. (b–d) Schematic overview of flagellar assembly. Expression of the master regulator gene *flrA* as well as those of other flagellar regulators (*flhFG*, *fliA*, *flgM*) and components of the basal body are FlrA-independent. The binding of FlhG or c-di-GMP to FlrA represses transcriptional activity. FlrA/RpoN activate the expression of additional genes. These include components of the basal body, hook, and first component of the filament. After assembly of the hook, the anti-sigma factor FlgM is exported and FliA can activate gene expression of late flagellar genes. These include the major flagellin *flaB* as well as the motor protein *motY*. Flagellar components labelled in red are independent of FlrA. Gene expression of components labelled in blue is activated by FlrA as well as RpoN and components labelled in purple are activated by FliA. For further explanations see the Discussion section.

a production of a significant subset of flagellar proteins, in particular those for the synthesis of the flagellar rod and hook structures. Accordingly, no functional flagella can be synthesized in the absence of these regulators in these species (Correa et al., 2000; Correa & Klose, 2005; Dasgupta et al., 2003; Klose & Mekalanos, 1998; Prouty et al., 2001). It has been proposed that, as shown for the *Campylobacter jejuni* FlgSR two-component system, FlrBC and FleSR form a regulatory checkpoint for completion of the MS-ring, the C-ring and the ft3SS, before the assembly of the following parts is initiated (Burnham et al., 2020). This checkpoint appears to be absent in *Shewanella* without negatively affecting flagellar synthesis. In *S. oneidensis*, the FlrBC is thought to be involved in regulating the abundance of the minor flagellin FlaA, which may influence the stiffness and geometry of the flagellar filament and, by this, the swimming

performance (Gao et al., 2018; Shi et al., 2014), which could not be observed in *S. putrefaciens* (Kühn et al., 2018). It is possible that *S. putrefaciens* FlrBC is activated and has a regulatory function under particular environmental conditions, but this remains to be shown.

An unexpected finding of our study was the number of flagellar proteins that were present at normal abundance also in the absence of FlrA/RpoN. Amongst these proteins were most, but not all, components of the ft3SS and two of the three building blocks forming the cytoplasmic C-ring (FliM and FliN). Also, three important regulators are amongst the constitutively produced flagellar proteins, FlhF, FlhG, and the sigma factor FliA (σ^{28}) along with its anti-sigma factor FlgM. For most of the corresponding genes or gene clusters, we could identify appropriate promoters. One possible explanation is that strict regulation of early building blocks has been lost during evolution except for some key elements, such as FliF, FliG and FlhA, to initiate the formation of the MS- and C-ring, and the ft3SS, respectively. This would, however, occur at the cost of the energy required for constant protein production. In addition, the number of identified promoters that are particularly present upstream of the regulator-encoding genes *flhF*, *flhG* and fliA rather pointed at a biological function of the FlrA-independent production. In this study, we could identify at least one of such possible functions, which is related to the regulator pair FlhF and FlhG.

The SRP-type GTPase FlhF and the MinD-like ATPase FlhG are implicated in flagellar positioning and number in a number of bacterial species (Altegoer & Bange, 2015; Kazmierczak & Hendrixson, 2013; Schuhmacher, Thormann, & Bange, 2015b). As in most other polarly flagellated species where FlhF has been studied, the latter is a positive regulator of flagellar synthesis and serves as a determinant for polar localization of the flagellum in *Shewanella* (Gao et al., 2015; Rossmann et al., 2015). In *S. putrefaciens*, deletion of *flhF* results in a lower number of flagellated cells with frequent flagellar misplacement away from the cell pole, whilst overproduction of FlhF gives rise to a polar flagellar bundle (Rossmann et al., 2015). In contrast, loss of *flhG* leads to polar hyperflagellation, whilst *flhG* overexpression significantly decreases flagellar formation (Blagotinsek et al., 2020; Schuhmacher, Rossmann, et al., 2015a). The current model for polarly flagellated bacteria suggests that an ATP-bound dimeric FlhF localizes to the cell pole and recruits FliF and/or facilitates assembly of the MS-ring as the earliest component of the bacterial flagellum in the cytoplasmic membrane (Green et al., 2009; Schuhmacher, Thormann, & Bange, 2015b). In *S. putrefaciens*, monomeric FlhG binds to the C-ring component FliM(N) and the complex travels to the nascent basal body (Schuhmacher, Rossmann, et al., 2015a). Upon assembly of FliM into the C-ring, FlhG is released, and, as a membrane-associated ATP-bound dimer, stimulates the GTPase activity of FlhF (Bange et al., 2011; Rossmann et al., 2015), resulting in FlhF monomerization and loss of polar localization. In addition, the ATP-bound FlhG dimer can interact with FlrA, which interferes with FlrA activity as an activator of the early flagellar transcription, thereby shutting down the synthesis of more earlier building blocks upon completion of C-ring assembly (Blagotinsek et al., 2020). Notably, FlrA stimulates FlhG ATPase activity which results in FlhG monomerization and loss of FlrA interaction. Accordingly, interference with any of these protein interactions or the protein ratios results in at least a partial loss of the proper regulation of flagellar counting and placement (Blagotinsek et al., 2020). To keep the number of polar flagella to one, the regulating partner switch has to be maintained also after the flagellar synthesis has been completed to prevent the initiation of the next round of flagellar formation. We have shown here that partial silencing of the promoters upstream of *flhG* results in cells with more than a single polar filament. We, therefore, propose that constitutive FlhG production is required to maintain a level of protein that balances initiation and prevention of forming additional flagella and thus renders the flagellar counter more robust. However, we so far do not know whether or not these newly identified promoters are constantly active or are themselves regulated in a way not depending on RpoN, FlrA, or FliA. It is possible the σ^{70} housekeeping sigma factor is responsible for promoter activity, but as the consensus binding region in *Shewanella* is poorly conserved (Shao et al., 2014) and as also further regulators maybe involved, this hypothesis warrants further studies. Similarly, it is also yet unknown whether the putatively RpoN-dependent promoters upstream of *fliA*, *fliK* and *flhF* require an enhancer-binding regulator other than FlrA or if they function—at a low level—without.

In addition, we were so far not able to sufficiently modify the predicted promoter regions in a way to suppress the permanent expression of the other regulator-encoding genes to address if this has any effect on flagellar synthesis and activity. Given the roles of FlhF in the flagellar placement and counting regulation, such a role is easily conceivable. Notably, also FliM(N), the C-ring-binding partners of FlhG, are amongst the constitutively produced proteins. It has been shown for *Escherichia coli* flagellar motors that FliMN is constantly exchanged in the rotating flagellar motor (Delalez et al., 2010, 2014). Thus, we hypothesize that a similar exchange in the *S. putrefaciens* motor would promote polar FlhG trafficking to maintain the regulatory circle as described, however, this remains speculative so far. A reason for FliA (σ^{28}) being constitutively produced (along with its anti-sigma factor FlgM) is not obvious as our results rather predict that the regulatory role of this sigma factor is, as expected, in initiating production of the main flagellin and enabling assembly of the T-ring to allow coupling of the stators to the motor to start rotation.

Taken together, our study demonstrates another level of regulation underlying the usual transcriptional hierarchy proposed for flagellar synthesis. It is yet unclear if this mode of regulation also applies to other bacteria. Transcriptional studies on different species identified genes that appear to be constitutively transcribed, e.g, the *fliHIJ* gene cluster in *Pseudomonas putida* (Leal-Morales et al., 2022) or *flgA* and *fliEFGHIJ* in *Vibrio campbellii* (Petersen et al., 2021). Also in *P. aeruginosa*, similar genes and operons as in *S. putrefaciens* (*fliLMNOPQR-flhB; fleN-fliA*) are only slightly regulated at the transcriptional level (Dasgupta et al., 2003), suggesting that the proteins may be permanently present. Also, a sigma 70-dependent promoter for *flhF* has been identified in *P. putida* (Kim et al., 1995). However, whilst numerous studies have addressed the transcriptional hierarchy of flagellar synthesis in an array of different bacterial species,

surprisingly little is known about the regulation of flagellar protein levels in the cells. A study comparing the proteome of *Xanthomonas oryzae* wild type to that of a FleQ (FlrA) mutant identified surprisingly few proteins related to flagella-mediated motility (Bae et al., 2018), indicating a significantly different regulation cascade in this species. We expect that more proteomic studies on flagellar regulation will uncover similar or additional principles of regulating flagellar synthesis and assembly. For monopolarly flagellated bacteria, such as *S. putrefaciens*, it is important that upon completion of the flagellar basal body, re-initiation of flagellar assembly of another flagellum is prevented, so the copy number of at least the key initial proteins, FliF, FliG, and FlhA, need to be sufficiently low to inhibit the start of another round of flagellar assembly. To this end, the production of these proteins could be extremely well controlled or the surplus of these key building blocks is rapidly cleared from the cell. How *S. putrefaciens* in particular and bacteria in common are achieving this is currently under investigation.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains, growth conditions and media

Strains of *Escherichia coli* and *Shewanella putrefaciens* CN-32 used in this study are listed in Table S3. *Shewanella* strains were grown at room temperature or 30°C in LB medium, *Escherichia coli* at 37°C in LB medium. The Medium of the 2,6-diaminopimelic acid (DAP)-auxotroph *E. coli* WM3064 were supplemented with DAP to a final concentration of 300 μM. When appropriate, selective media were supplemented with 10% (w/v) sucrose, 50 mg/ml kanamycin, 2% (w/v) L-arabinose or 40 ng/ml anhydrotetracyclin (AHT). Soft agar plates were prepared with 0.25% (w/v) select agar and LB.

4.2 | Vector and strain constructions

All plasmids and oligonucleotides used in this study are listed in Tables S4 and S5. Construction of plasmids was performed using Gibson assembly (Gibson et al., 2009). Generation of markerless *in-frame* deletions and chromosomal integrations of gene variants or fusions in *S. putrefaciens* was carried out using the suicide vector pNPTS 138-R6K with EcoRV as previously described (Lassak et al., 2010). In order to overproduce the flagellar main regulator FlrA (Sputcn32_2580) the vector pBTOK (Rossmann et al., 2015) or a chromosomal integration into the major arabinose degradation pathway as previously described (Mayer et al., 2021) were used. For analysis of flagellar promoter activity in *S. putrefaciens*, a transcriptional terminator cassette and the *luxCDABE* operon of *Photorhabdus luminscence* were amplified from pBBR1-MCS5-TT-RBS-lux (Gödeke et al., 2011) and cloned into the vector pBBR1-MCS2, using SacI and KpnI, respectively, yielding vector pBBR1-MCS2-TT-RBS-lux. The promotor regions to be analyzed were inserted upstream of the lux operon using XbaI and BamHI. Vectors were introduced into the appropriate strains via conjugation using *E. coli* WM3064 as a donor.

4.3 | Immunoblot analysis

The regulation of protein amount was determined by western blot analysis. Collection of protein samples, protein separation and immunoblot detection were carried out as previously described (Blagotinsek et al., 2020; Bubendorfer et al., 2012; Schuhmacher, Rossmann, et al., 2015a).

4.4 | Quantitative RT-PCR

The total RNA of exponentially growing *Shewanella* cells were isolated as previously described (Blagotinsek et al., 2020). Residual DNA was removed with the Turbo DNA-free Kit (Invitrogen) according to the manufacturer's protocol. For the quantitative Real-Time PCR (qPCR) the CFX Connect Real-Time System (Bio-Rad) and white strips of low profile tubes with ultra clear caps (Thermo Fisher Scientific) were used for PCR amplification. The qPCR was performed as previously described (Blagotinsek et al., 2020). Oligonucleotides (Sigma-Aldrich) used for qPCR are listed in Table S5. Primer efficiencies and relative transcript levels were calculated according to Pfaffl (Pfaffl, 2001). Ct values for each gene of interest were normalized against the Ct value of Sputcn32_2070 (*gyrA*) and computed relative to the respective control.

4.5 | Luciferase assay

Measuring transcriptional activities of promoters with fusions to the *luxCDABE* operon was performed as previously described (Bubendorfer et al., 2012). *Shewanella* strains were diluted to an OD_{600} 0.02 from overnight culture and grown under aerobic conditions to an OD_{600} 0.5–0.6 at 30°C. Exponential growing cells are transferred in 160 μl aliquots to a white 96-well polypropylene microliter plate (Greiner). Luminescence emission was measured in technical and biological triplicates using a Tecan Infinite M200 plate reader (Tecan). The relative luminescence units (RLU) were calculated by dividing the luminescence intensity by its corresponding OD_{600} value to normalize the luminescence to OD_{600} 1.

4.6 | 5´RACE-PCR

For identification of transcriptional start sites (TSS) were 5´RACE PCR performed. DNA-free RNA of *Shewanella* strains with the promoters to be analyzed on the pBBR1-MCS2-TT-RBS-lux plasmid were isolated from exponentially growing cultures. The further procedure was performed using the 5′/3´RACE PCR Kit, 2nd Generation (Roche) according to the manufacturer's protocol. The

amplification of dA-tailed cDNA in a first and second PCR was performed with Biozym Taq DNA Polymerase (Biozym) according to the manufacturer's protocol. The corresponding oligonucleotides are listed in Table S5. Ligation of 1 µl 5´RACE PCR products were performed with the Qiagen PCR cloning kit (Qiagen) according to the manufacters protocol. Inserts of plasmids were sequenced with oligonucleotide pUCM13-52 by Sanger sequencing (Microsynth seqlab).

4.7 | Flagellar staining and microscopy

Fluorescent staining of polar flagellar filaments (CN-32 $FlaAB_{cys}$) was carried out as previously described (Guttenplan et al., 2013; Kühn et al., 2017; Rossmann et al., 2019) using CF488A maleimide (Sigma-Aldrich). Fluorescence images were recorded by a DMI6000 B inverse microscope (Leica) equipped with a pco.edge sCMOS camera (PCO) and an HC PL APO 100x/1.40 Oil PH3 phase contrast objective using the VisiView software (Visitron Systems GmbH). Images were further processed using the ImageJ-based Fiji tool (Schindelin et al., 2012) and Affinity Designer 1.7v (Serif).

4.8 | Motility assay

The spreading ability of *Shewanella* strains in semi-solid environments was analyzed by placing 2.5 µl of an exponentially grown culture on a soft agar plate. After incubation for 16 h at room temperature, the plates were scanned for documentation using an Epson Perfection V700 Photo Scanner (Epson).

4.9 | RNA sequencing and data analysis

Bacteria were grown in 10 ml LB to early stationary phase (OD600 0.5, 30°C, 180 rpm). RNA was extracted using the RNeasy Mini Kit (Qiagen) and Qiashredder columns (Qiagen) according to the manufacturer's instructions. The quality of the obtained RNA was checked using the RNA Nano Kit on an Agilent Bioanalyzer 2100 (Agilent Technologies). The removal of ribosomal RNA was performed using the Ribo-Zero Bacteria Kit (Illumina) and cDNA libraries were generated with the ScriptSeq v2 Kit (Illumina). The samples were sequenced in single-end mode on an Illumina HiSeq 2500 device. Sequencing was conducted on RNA obtained from two independent growth experiments. Raw reads were trimmed using the tool 'cutadapt' (version 3.5) (Martin, 2011) with customized settings (-- nextseq-trim = 20; g 'TTTTT;min_overlap = 1'; -m 36). Mapping was performed with 'bowtie2' (version 2.3.5.1) (Langmead & Salzberg, 2012) with the settings '--very-sensitive-local -I 100 -X 1200' and with the NC_009438.1 (*Shewanella putrefaciens* CN-32) genome as a reference. Reads per gene were extracted with the tool 'featureCounts' (version 2.0.1) (Liao et al., 2014) (Figure S6b). Data analysis including the MDS plot was performed with the R package edgeR (v.3.32.1) (Robinson et al., 2010) (Figure S6a). qPCR was conducted on *fliF* and *fliM* as another control (Figure S6c). Significant regulation was accepted at a $\log_2$ fold change of less than −1 or greater than 1 and a *p*-value of less than 0.05.

4.10 | Mass spectrometry

Proteomic analyses were performed as described recently (Sander et al., 2019). In short, cell pellets were lyzed by 2% sodium laroyl sarcosinate (SLS) and heat. Following reduction and alkylation, 50 µg of extracted protein was digested by trypsin. Post digest the detergents were precipitated by adding acid and peptides were desalted using C18 solid phase extraction. 1 µg of peptides were then analyzed by liquid chromatography-mass spectrometry carried out on a Q-Exactive Plus instrument connected to an Ultimate 3000 RSLC nano. Peptide separation was performed on a reverse-phase HPLC column (75 µm x 42 cm) packed in-house with C18 resin (2.4 µm, Dr. Maisch). The following separating gradient was used: 98% solvent A (0.15% formic acid) and 2% solvent B (99.85 acetonitrile, 0.15% formic acid) to 25% solvent B over 105 min and 35% solvent B for additional 35 min at a flow rate of 300 nl/min. The data acquisition mode was set to obtain one high-resolution MS scan at a resolution of 70,000 full width at half maximum (at m/z 200) followed by MS/MS scans of the most intense ions. The dynamic exclusion duration was set to 30 s. The ion accumulation time was set to 50 ms for MS and 50 ms at 17,500 resolution for MS/MS. The automatic gain control was set to 3×10^6 for MS survey scans and 1×10^5 for MS/MS scans.

Label-free quantification (LFQ) of the data was performed using Progenesis QIP (Waters) and SafeQuant. The strategy has been further described in (Ahrné et al., 2013; Glatter et al., 2012). Significant regulation was accepted at a $\log_2$ fold change of less than −1 or greater than 1 and a *p*-value of less than 0.05.

AUTHOR CONTRIBUTIONS

Meike Schwan: Conceptualization; Writing – original draft; Writing – review & editing; Investigation. **Ariane Khaledi:** Investigation; Writing – review & editing. **Sven Willger:** Investigation; Data curation; Writing – review & editing. **Kai Papenfort:** Validation; Writing – review & editing; Resources; Funding acquisition. **Timo Glatter:** Investigation; Validation; Data curation; Writing – review & editing. **Susanne Häußler:** Conceptualization; Funding acquisition; Writing – review & editing; Supervision; Resources. **Kai M. Thormann:** Conceptualization; Funding acquisition; Writing – original draft; Writing – review & editing; Supervision; Resources.

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CONFLICT OF INTEREST

The authors declare that no financial, personal, or professional interests have influenced the work.

DATA AVAILABILITY STATEMENT

The RNA sequencing data have been deposited at GEO under the accession number GSE207531. All other data are either included in the manuscript or available upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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