Spatiotemporal control of FlgZ activity impacts *Pseudomonas aeruginosa* flagellar motility

FlgZ activity impacts flagellar motility

Sarina Bense[1], Sebastian Bruchmann[1,†], Anika Steffen[2], Theresia E. B. Stradal[2], Susanne Häussler[1,§], Juliane Düvel[1,‡,§]

[1]Helmholtz Center for Infection Research, Molecular Bacteriology, Inhoffenstr. 7, 38124 Braunschweig, Germany

[2]Helmholtz Center for Infection Research, Cell Biology, Inhoffenstr. 7, 38124 Braunschweig, Germany

[†]Present address: Wellcome Sanger Institute, Pathogen Genomics, Hinxton, Cambridgeshire, CB10 1SA, UK

[‡]Present address: Robert Koch Institute, Division of Enteropathogenic Bacteria and Legionella (FG11), Burgstraße 37, 38855 Wernigerode, Germany

[§]Susanne Häussler and Juliane Düvel are joint senior authors

*Corresponding author: susanne.haeussler@helmholtz-hzi.de, +49 531 6181 3000

Keywords

c-di-GMP, flagellar motility, motor brake, FlgZ, Pseudomonas

Summary

The c-di-GMP-binding effector protein FlgZ has been demonstrated to control motility in the opportunistic pathogen *Pseudomonas aeruginosa* and it was suggested that c-di-GMP-bound FlgZ impedes motility via its interaction with the MotCD stator. To further understand how motility is downregulated in *P. aeruginosa* and to elucidate the general control mechanisms operating during bacterial growth, we examined the spatiotemporal activity of FlgZ. We re-annotated the *P. aeruginosa flgZ* open reading frame and demonstrate that FlgZ-mediated downregulation of motility is fine-tuned via three independent mechanisms. First, we found that *flgZ* gene is transcribed independently from *flgMN* in stationary growth phase to increase FlgZ protein levels in the cell. Second, FlgZ localizes to the cell pole upon c-di-GMP binding and third, we describe that FimV, a cell pole anchor protein, is involved in increasing the polar localized c-di-GMP bound FlgZ to inhibit both, swimming and swarming motility. Our results shed light on the complex dynamics and spatiotemporal control of c-di-GMP dependent bacterial motility phenotypes and on how the polar anchor protein FimV, the motor brake FlgZ and the stator proteins function to repress flagella-driven swimming and swarming motility.

Introduction

The ubiquitous second messenger bis-(3'-5')-cyclic di-GMP (c-di-GMP) has been demonstrated to regulate the transition between a motile planktonic and a sessile biofilm-associated lifestyle in a wide range of bacteria, including the opportunistic pathogen *Pseudomonas aeruginosa*. Elevated concentrations of intracellular c-di-GMP inhibit motility and virulence and lead to the initiation of biofilm formation, whereas low levels of c-di-GMP promote the motile lifestyle (Jenal and Malone, 2006; Hengge, 2009; Römling *et al.*, 2013). The intracellular c-di-GMP level is tightly controlled by the opposing activities of two classes of enzymes. The diguanylate cyclases (DGCs) produce c-di-GMP from two molecules of guanosine triphosphate (GTP). The degradation of c-di-GMP into linear pGpG or into two GMP molecules is catalyzed by EAL or HD-GYP domain containing phosphodiesterases (PDE), respectively (Ross *et al.*, 1987; Paul *et al.*, 2004; Ryjenkov *et al.*, 2005; Schmidt *et al.*, 2005; Christen *et al.*, 2005; Ryan *et al.*, 2006). The activity of these enzymes is regulated by sensory input domains that respond to a broad spectrum of environmental signals such as oxygen, light, surface attachment and antibiotics (Hoffman *et al.*, 2005; Tarutina *et al.*, 2006; Güvener and Harwood, 2007; Barends *et al.*, 2009; Tuckerman *et al.*, 2009; Okegbe *et al.*, 2017). C-di-GMP was first identified as an allosteric activator of a cellulose synthase found in *Gluconacetobacter xylinus* (Ross *et al.*, 1987). Since then, several c-di-GMP effectors were identified and functionally characterized, including proteins containing PilZ domains or degenerated GGDEF or EAL domains, but also effectors with new binding domains (Chou and Galperin, 2016). Also, several c-di-GMP responsive riboswitches have been described (Sudarsan *et al.*, 2008; Lee *et al.*, 2010).

Conformational changes in c-di-GMP binding domains enable the activity regulation of targeted effectors by c-di-GMP. C-di-GMP binding thus impacts on diverse functions such as enzymatic activities, protein-protein interactions, transcription and translation (Valentini and Filloux, 2016). A well-studied example of a PilZ domain effector protein is YcgR of *Escherichia coli*. YcgR was found to regulate swimming motility by its interaction with the flagellar motor proteins FliG and MotA (Paul *et al.*, 2010; Boehm *et al.*, 2010; Fang and Gomelsky, 2010). This interaction depends on c-di-GMP binding to YcgR and disturbs the electrostatic interaction between MotA and FliG, thereby impacting torque generation and swimming speed. In *P. aeruginosa*, the PilZ domain protein PA3353 displays sequence similarity to YcgR. We have previously used agarose-bound c-di-GMP to capture new c-di-GMP effectors in *P. aeruginosa.* Among the proteins that were repeatedly pulled-down was PA3353, which bound c-di-GMP with high affinity (Düvel *et al.*, 2012). PA3353 was found to downregulate motility in *P. putida* and *P. aeruginosa* and thus was annotated as *flgZ* (Martínez-Granero *et al.*, 2014; Baker *et al.*, 2016). Furthermore, FlgZ has been shown to interact directly with the stator protein MotC in *P. aeruginosa* to repress swarming upon c-di-GMP binding by preventing engagement of MotCD with the rotor (Baker *et al.*, 2016).

To further characterize the functional role of FlgZ in *P. aeruginosa*, we analyzed the dynamic changes of *flgZ* transcription and applied a suppressor mutant screen aiming at the identification of additional FlgZ interaction partners that modulate its activity. We found that transcription of *flgZ* is decoupled in early stationary phase and that polar localization of FlgZ is not only dependent on c-di-GMP binding but is also impacted by the presence of the putative polar anchoring protein FimV. Our results shed light on the complex dynamics and spatiotemporal control of c-di-GMP dependent bacterial motility phenotypes.

Results

Growth phase dependent transcription and translation of flgZ

C-di-GMP levels increase during entry into stationary phase (Pesavento *et al.*, 2008). We therefore wondered whether the levels of the c-di-GMP binding protein FlgZ increase accordingly. Indeed, as demonstrated by Western blot analysis using an anti-FlgZ antibody, the protein levels of FlgZ were elevated in stationary growth as opposed to exponential growth phase (Fig. 1A). Re-analysis of previously recorded RNA-sequencing data (Dötsch *et al.*, 2012) revealed that also transcription of the *flgZ* gene was enhanced in stationary phase (Fig. 1B). However, the gene expression levels of *flgM* and *flgN,* both of which are localized on the same operon as *flgZ* (Fig. 1C), were comparable in exponential and stationary phase. To investigate whether *flgZ* is co-transcribed with *flgMN* or whether there is an (additional) transcriptional start site upstream of *flgZ*, we performed reverse transcription based PCR experiments. During exponential growth *flgZ* is part of a polycistronic *flgMNZ* transcript (Fig. 1D). However, in early stationary phase *flgZ* is transcribed independently of *flgMN* (Fig. 1C+D). 5´RACE (rapid amplification of cDNA ends) experiments revealed two transcriptional start sites (TSS) upstream of *flgM*, the first gene of the *flgMNZ* operon (Fig. 2). One was located 30 bp upstream of the *flgM* open reading frame (ORF) and has been previously described in different *P. aeruginosa* strains (Frisk *et al.*, 2002; Dötsch *et al.*, 2012; Wurtzel *et al.*, 2012). A second TSS was found at position -83 bp relative to the *flgM* ORF. 5´RACE experiments targeting *flgZ*-specific transcripts revealed a transcription start site positioned 17 bp downstream of the annotated start of the *flgZ* gene (Fig. 2). This TSS was identified in 5´RACE experiments based on RNA, which was isolated from exponential and from early stationary growth phase, respectively. Thus, there are polycistronic transcripts (prevalent in exponential growth phase) which encode FlgZ as well as monocistronic *flgZ t*ranscripts (prevalent in both, exponential and stationary growth phase), in which the *flgZ* gene is transcribed independently from *flgMN*.

Revisiting the open reading frame of flgZ

As the 5´RACE experiments uncovered the *flgZ* transcriptional start site 17 bp downstream of the annotated gene start site, we re-analyzed the nucleotide sequence of the *flgZ* gene in order to identify potential alternative start codons. Indeed, 26 bp downstream of the *flgZ* TSS a GTG codon that could serve as translational start site was found. This possible start codon is preceded by an AG rich region potentially representing a ribosomal binding site (RBS) (Fig. 2). Translation from this GTG (42-45 bp downstream of the annotated *flgZ* gene start) would not alter the reading frame and would produce a FlgZ protein that is 14 amino acids shorter than the previously annotated protein. Fig. 3 shows an alignment of the nucleotide sequence of PAO1_*flgZ* with orthologues from nine other *Pseudomonas* species. The gene start of all other *flgZ*-orthologues was annotated at the position that corresponds to the alternative GTG start (Fig. 3A). To experimentally identify the translational start site of FlgZ, we aimed for N-terminal protein sequencing. Therefore, we constructed a PAO1 strain carrying the *flgZ* allele with a chromosomal insertion of a 6×His-tag coding sequence at the 3´ end. The FlgZ-His6 protein was isolated from cells that had reached early stationary phase and was subsequently purified by affinity chromatography. N-terminal protein sequencing by Edman degradation revealed the amino acid sequence PNPFVEEAGP as the beginning of the FlgZ protein (Fig. 3B). This finding is in agreement with an open reading frame starting at nucleotide position +42 relative to the previously annotated *flgZ* gene.

FliA and RpoN are involved in flgZ *expression*

When revisiting our RNA-sequencing data, which have been generated to describe the regulons of a diverse set of alternative sigma factors (Schulz *et al.*, 2015), we found a positive regulation of *flgMNZ* by FliA (Fig. 4A). Furthermore, *flgZ* expression was significantly downregulated in an *rpoN* mutant, whereas the transcript levels of *flgM* and *flgN* were slightly, but not significantly decreased. This is in agreement with previous studies demonstrating that both alternative sigma factors impact motility phenotypes (Arnosti and Chamberlin, 1989; Totten *et al.*, 1990; Starnbach and Lory, 1992). The RNA-sequencing data (Schulz *et al.*, 2015) also revealed that neither *flgMN* nor *flgZ* expression was subject to regulation by the stationary phase sigma factor RpoS, despite the observed increased *flgZ* expression in stationary phase.

To confirm these observations and to analyze if the region around the *flgZ* start displays promoter activity, we constructed a transcriptional fusion of the putative *flgZ* promoter region with *luxCDABE* genes and introduced the construct into the PA14 wild type, as well as in *ΔfliA*, *ΔrpoN* and *ΔrpoS* mutant strains. A clear bioluminescence signal was observed in the PA14 wild type strain and the *ΔrpoS* mutant indicating that RpoS is not required for *flgZ* transcription (Fig. 4B). In contrast, expression of the reporter construct was significantly reduced in the *ΔfliA* and the *ΔrpoN* mutant, indicating that both sigma factors positively impact on *flgZ* transcription.

Sigma factor binding sites as identified by ChIP-seq experiments and motif searches were previously published by our group (Schulz *et al.*, 2015). A FliA binding site upstream of *flgM* was identified and an RpoN binding site upstream of *flgZ*. The ChIP-seq data revealed binding of FliA upstream of *flgMNZ*, but no RpoN signal was detected (Schulz *et al.*, 2015).

C-di-GMP binding of FlgZ is important for the negative impact on motility

With the aim to analyze the effect of FlgZ on motility behavior of *P. aeruginosa* PAO1 in more detail, we selected a *flgZ* transposon mutant (Jacobs *et al.*, 2003) and also overexpressed *flgZ*. We furthermore constructed a strain in which FlgZ was overproduced as a non-c-di-GMP binding variant. Therefore, we exchanged the two arginine residues of the RXXXR binding motif of the FlgZ PilZ domain for alanine (R126A and R130A) and tested this FlgZ protein variant for c-di-GMP binding. *In vitro* produced proteins containing either the native RNAYR motif or the mutated ANAYA motif were spotted on a nitrocellulose membrane and incubated with fluorescently labeled c-di-GMP. While the wild type variant with the RNAYR motif clearly showed c-di-GMP binding, no c-di-GMP binding was observed for the FlgZ ANAYA variant (Fig. 5A).

The *flgZ* mutant displayed a slight but significant increase in swimming motility compared to the wild type, whereas overexpression of *flgZ* significantly impaired swimming motility (Fig. 5B). The impact of *flgZ* deletion on swarming motility was less consistent under our experimental conditions. However, *flgZ* overexpression clearly affected the cells ability to swarm (Fig. 5C). Motility assays with strains overexpressing the non-c-di-GMP binding variant revealed that binding of c-di-GMP to FlgZ is required to downregulate both, swimming and swarming motility.

(Partial) deletion of fimV *overrides the negative impact of* flgZ *overexpression on motility*

We were interested in identifying additional bacterial factors that are involved in the inhibition of bacterial motility upon c-di-GMP binding to FlgZ. We therefore overexpressed *flgZ* in PAO1 and

screened for suppressor mutants, in which the impaired motility is relieved. For this purpose the *flgZ* overexpressing strain was repeatedly transferred on swarming agar plates on a daily basis. After seven rounds, we identified a suppressor mutant exhibiting wild type swarming motility despite *flgZ* overexpression (Fig. 6A). Whole genome sequencing of two colonies from this last swarming plate revealed a deletion at positions 3´497´669 to 3´497´881 in the PAO1 genome in both isolates. This deletion was located within the *fimV* gene (PA3115) and corresponds to the deletion of amino acids 109 – 179 in the FimV protein. Previous studies on *P. aeruginosa* FimV uncovered a role in type IV pilus assembly. Thereby the ability of FimV to bind to peptidoglycan was demonstrated to be crucial for FimV function (Semmler *et al.*, 2000; Wehbi *et al.*, 2011). The partial deletion in *fimV* that was found in our suppressor mutant includes parts that encode the peptidoglycan binding motif, suggesting a functional impairment of the FimV protein in the suppressor mutants. HubP, the orthologue of FimV in *V. cholera* and *S. putrefaciens*, was shown to be a polar landmark protein required for directing the chromosome segregation and chemotactic machinery as well as the flagellar system to the cell pole (Yamaichi *et al.*, 2012; Rossmann *et al.*, 2015).

We constructed a mutant harboring the partial deletion of *fimV* as well as a mutant with a complete *fimV* knock out and analyzed the motility behavior (Fig. 6B). Clearly, partial deletion as well as complete knock out of *fimV* counteracted the negative impact of *flgZ* overexpression on swarming motility and enabled a faster migration on swarming motility plates as compared to the wild type overexpressing *flgZ*. The complete *fimV* knock out mutant was selected for further analysis. The *fimV* knockout mutant displayed an increased swarming and also - albeit to a lesser extent - increased swimming motility as compared to that of the wild type (Fig. 6 C+D). Overexpression of c-di-GMP binding FlgZ clearly reduced swimming and swarming motility in the wild type, while the non-c-di-GMP binding *flgZ* variant ANAYA did not. In the *fimV* mutant overexpression of c-di-GMP binding FlgZ clearly also reduced swimming and swarming motility. However, overexpression of the non-c-di-GMP binding *flgZ* variant ANAYA did restore swimming motility in the *fimV* mutant to wild-type levels, but not swarming motility. Thus, overexpression of *flgZ* can inhibit motility despite the lack of *fimV*. With regard to swarming motility this is even possible if a non-c-di-GMP binding variant of FlgZ is overexpressed.

Number and position of the flagellum is not altered in the fimV *deletion mutant*

A previous publication described an interaction of HubP, the FimV orthologue of *Vibrio cholerae*, with the two motility proteins FlhF and FlhG and a role of HubP for the polar localization of FlhG (Yamaichi *et al.*, 2012). These two motility proteins have been implicated to play a role in the regulation of flagellar number and localization in *V. cholerae*, *P. aeruginosa* and other bacterial organisms (Dasgupta *et al.*, 2000; Dasgupta and Ramphal, 2001; Correa *et al.*, 2005; Murray and Kazmierczak, 2006). We therefore investigated flagellation of the *ΔfimV* mutant by transmission electron microscopy. Stationary phase grown cells as well as cells from the outer rim of a swarming colony were analyzed. Under both conditions, the majority of *P. aeruginosa* PAO1 wild type and *ΔfimV* mutant cells expressed a single polar flagellum (Fig. 7A). Thus, it is unlikely that an altered flagellation of the *ΔfimV* mutant is responsible for the altered motility behavior.

Decreased amount of polar localized GFP-FlgZ in the fimV *deletion mutant*

As orthologues of FimV have been described to anchor proteins to the cell pole (Yamaichi *et al.*, 2012), we monitored localization of a plasmid encoded GFP-FlgZ fusion in the wild-type and the *fimV*

mutant by fluorescence microscopy. GFP-FlgZ displayed polar puncta in a fraction of cells of both, the wild type and the *ΔfimV* mutant strain (Fig. 7B). The quantification of the amount of polar localized GFP-FlgZ revealed a significant decrease in the *ΔfimV* mutant compared to the wild type indicating that FimV is important for the polar localization of FlgZ (Fig. 7C). Expression of a GFP-FlgZ fusion with the non-c-di-GMP binding motif ANAYA significantly decreased the fraction of polar localized FlgZ in the wild type but not in the *ΔfimV* deletion strain. We conclude that FimV is partially responsible for the polar localization of FlgZ and that the binding of c-di-GMP to FlgZ promotes polar localization of FlgZ.

Discussion

The FlgZ orthologue YcgR is a c-di-GMP binding protein that inhibits motility in *E. coli* through a brake mechanism that impedes torque generation of the flagellar motor in a c-di-GMP concentration dependent manner (Boehm *et al.*, 2010). To further understand how motility is down-regulated in *P. aeruginosa* and to elucidate the general control mechanisms operating during bacterial growth, we examined the spatiotemporal activity of the PilZ domain protein FlgZ. We re-annotated the *P. aeruginosa flgZ* ORF and show that FlgZ impairs swimming and swarming motility in *P. aeruginosa.* Thereby, downregulation of motility is fine-tuned via three mechanisms: i) controlled growth phase dependent transcription of *flgZ*, ii) c-di-GMP dependent localization of FlgZ to the cell pole and iii) FimV dependent anchoring of FlgZ-c-di-GMP at the cell pole.

We demonstrate that the three genes *flgMNZ* can be transcribed as one operon, but *flgZ* can also be transcribed from its own transcriptional start site. This decouples *flgMN* transcription from *flgZ* transcription. FlgM and FlgN are both involved in biosynthesis and assembly of the flagellum, and thus would be expected to be expressed during exponential phase, in which cells divide and synthesize new flagella. In contrast, FlgZ regulates the flagellar function. When cells are starting to slow down and attach to surfaces to initiate formation of a biofilm, FlgZ is required to reduce flagellar mediated motility. Coupling of *flgMN* to *flgZ* transcription during exponential growth phase thus seems reasonable, because FlgZ can only function if a flagellum is present. Nevertheless, in early stationary phase we observed a decoupling of *flgZ* and *flgMN* transcription. At elevated c-di-GMP levels FlgZ activity is needed to shut down motility so that cells become sessile.

Transcription of *flgZ* is induced in stationary growth phase and is positively influenced by the activity of the two alternative sigma factors FliA and RpoN. FliA, the motility sigma factor, is involved in the transcription of chemotaxis and motility genes (Arnosti and Chamberlin, 1989; Starnbach and Lory, 1992), whereas RpoN controls a broader spectrum of genes, e.g. genes involved in nitrogen metabolism, virulence but also chemotaxis, motility and attachment (Gussin *et al.*, 1986; Ishimoto and Lory, 1989; Totten *et al.*, 1990; Cai *et al.*, 2015). A previous study revealed a four-tired hierarchy of transcriptional regulation of flagellar biosynthesis genes, which involved both FliA and RpoN (Dasgupta *et al.*, 2003). *FlgMNZ* transcription has been demonstrated before to be regulated by FliA and RpoN in *P. aeruginosa* PAK (Frisk *et al.*, 2002). In *P. putida,* expression of the *flgMNZ* genes is controlled by an RpoN-dependent read-through of upstream *flgA* transcription (Wirebrand *et al.*, 2018). A cooperation of FliA and RpoN was also found in our previous study on the direct crosstalk between alternative sigma factor regulons in *P. aeruginosa* (Schulz *et al.*, 2015). RpoN was identified as the sigma factor being most involved in direct crosstalk with other sigma factors and a set of 43 *P. aeruginosa* genes were activated by both, FliA and RpoN, respectively.

Analysis of the functional role of FlgZ, which we found to be 14 amino acids shorter than previously predicted, revealed that binding of c-di-GMP to FlgZ impairs swimming and swarming motility in *P. aeruginosa* PAO1. A negative impact of a respective FlgZ orthologue on swimming motility was demonstrated before for *P. putida* but was not observed in *P. fluorescens* (Martínez-Granero *et al.*, 2014). It has furthermore previously been demonstrated that swarming motility is repressed in *P. aeruginosa* PA14 via interaction of c-di-GMP bound FlgZ with MotC, a component of the flagellar stator complex (Baker *et al.*, 2016). Here, we show that the ability to bind c-di-GMP is also required for the negative impact on *P. aeruginosa* swimming motility. Furthermore, localization studies using a fluorescent FlgZ-reporter demonstrated that FlgZ is located at the cell pole and that localization of

FlgZ is less polar when the c-di-GMP binding motif is mutated. This indicates that binding of c-di-GMP promotes polar localization of FlgZ. However, polar localization of FlgZ was not only affected by c-di-GMP binding, as we also found significantly more polar c-di-GMP bound FlgZ in *P. aeruginosa* strains harboring an intact *fimV* gene. Accordingly, a deletion of *fimV* counteracted the negative impact of *flgZ* on motility.

Taken together, we found that c-di-GMP bound FlgZ localizes to the cell pole which is further positively influenced by the presence of the membrane anchor protein FimV. This indicates that both proteins are part of a polar macromolecular complex involved in the interaction of FlgZ with the stator protein MotC to repress flagella function. Upon c-di-GMP binding FlgZ prevents engagement of MotCD with the rotor in *P. aeruginosa* (Baker *et al.*, 2016). Clearly, more *in vivo* and *in vitro* studies are required in order to fully appreciate the contribution of polar proteins, including the stator proteins MotAB and MotCD, as players of flagella driven swimming as well as swarming motility.

Experimental procedures

Bacterial strains and growth conditions

Strains used in this study are summarized in Table S1. *Escherichia coli* and *Pseudomonas aeruginosa* strains were grown at 37 °C and 180 rpm in lysogeny broth (LB). If required, antibiotics were added to the growth medium at the following concentrations: 100 µg ml^{-1} ampicillin or 15 µg ml^{-1} gentamicin for *E. coli* and 400 µg ml^{-1} carbenicillin or 50 µg ml^{-1} gentamicin for *P. aeruginosa*.

DNA manipulation and strain construction

All plasmids used in this study are described in Table S2. Oligonucleotides used for plasmid and strain construction, but also for reverse transcriptase PCR and 5´RACE are listed in Table S3. In general, DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989) using appropriate kits (Qiagen) and enzymes (Thermo Fisher Scientific, Agilent Technology, New England Biolabs). Plasmids were constructed in a restriction and ligation approach. *P. aeruginosa* received plasmids either by electroporation (Choi *et al.*, 2006) or conjugation from *E. coli*. The complete *fimV* knock out mutant, the partial deletion of *fimV* and the *flgZ-his6* substitution at its native locus were constructed by allelic exchange using the pEX18Ap plasmid. The pEX18Ap constructs were conjugated into *P. aeruginosa* using the *E. coli* S17-1 strain. After conjugation into *P. aeruginosa* single crossovers were selected on LB supplemented with carbenicillin and afterwards double crossovers were counter-selected on LB supplemented with 10 % (w/v) sucrose. To generate a fluorescent FlgZ fusion protein, FlgZ was N-terminally tagged with GFP using a linker sequence of six amino acids: Ala-Gly-Gly-Ser-Gly-Ala. The GFP is an improved GFP variant with better folding properties, a higher solubility and an enhanced fluorescent intensity. Originally this GFP variant was called GFPmut2 and consists of three amino acid substitutions (S65A, V68L, S72A) (Cormack *et al.*, 1996). Point mutations were introduced to *flgZ* by site directed mutagenesis using the QuikChange II – Site-directed mutagenesis Kit (Agilent Technology) according to manufacturer´s instructions. All generated constructs were verified by sequencing (Eurofins Genomics).

RNA isolation, cDNA synthesis and Reverse Transcription-PCR (RT-PCR)

Total RNA was isolated by using the RNeasy® Mini Kit (Qiagen) in combination with QIAshredder™ (Qiagen) according to the manufacturer's instructions. Remaining DNA was removed by DNase treatment (DNA-free™ Kit DNase Treatment & Removal, ambion). The Superscript III™ Reverse Transcriptase (Invitrogen) and Random Primers (Invitrogen) were used for cDNA synthesis according to the manufacturer´s instructions with a slight modification. The reaction mixture was incubated at 50 °C for 2 h for cDNA synthesis. An RNase H (NEB) digestion was carried out to degrade the RNA strand of the RNA-cDNA hybrid. The cDNA was purified with the QIAquick® PCR Purification Kit (Qiagen) following manufacturer´s instruction. All PCR reactions were performed with the Herculase II Fusion Polymerase (Agilent) following the manufacturer´s instruction.

5´ RACE (Rapid amplification of cDNA ends)

5´ RACE experiments were performed as previously described (Dötsch *et al.*, 2012). Briefly, target gene specific primers (GSP1) were used for cDNA synthesis. A poly A-tail was added to the 3´ end of the synthesized cDNA by using the terminal deoxynucleotide transferase (TdT) (NEB) following manufacturer's instructions. PCR amplification of the target cDNA was carried out in a half-nested

PCR in order to increase the specificity. Therefore, a PCR setup containing three primers was used. The first primer contained a 3´ poly-T sequence that anneals to the poly-A-tail of the cDNA fragments. In addition, this primer harbors an anchor sequence enabling hybridization of a 5´ anchor primer. The actual amplification was based on this 5´ anchor primer and a second target gene specific primer (GSP2) binding to a sequence upstream of the sequence of GSP1. Hybridization of the poly-T-tailed primer to the cDNA occurred at 48 °C for 2 min followed by an elongation step at 72 °C for 40 min. The PCR amplification was performed with an annealing temperature of 59 °C and an elongation time of 15 s for 30 cycles. After purification of the PCR products transcriptional start sites were identified by sequencing (Eurofins Genomics).

Promoter activity assays

The *flgZ* promoter region from -51 to +32 (according to the annotated start of *flgZ*) was fused upstream of the *lux* genes of plasmid pBBR1-MCS5-TT-RBS-*lux* (Gödeke *et al.*, 2011). The resulting plasmid pBBR1-MCS5-TT-p*flgZ*-RBS-*lux* was introduced in PA14 wild type and the sigma factor knock out mutants PA14 *ΔfliA*, PA14 *ΔrpoN* and PA14 *ΔrpoS* (Schulz *et al.*, 2015) by electroporation as previously described (Choi *et al.*, 2006). Bioluminescence was measured in the Enspire® Multimode Plate Reader (Perkin Elmer, USA). The obtained luminescence signal was normalized to the optical density and the luminescence signal of the empty vector control.

Western blot analysis of FlgZ protein levels

Protein levels of FlgZ at different stages of bacterial growth were analyzed by western blot with a FlgZ specific antibody. The primary anti-FlgZ antibody was isolated from blood serum of rabbits immunized with a peptide consisting of the last 12 amino acids (REARRFEKDELF) of FlgZ by affinity purification. Cultures of the wild type and the *ΔfimV* mutant were grown to exponential and stationary growth phase. Aliquots of $2*10^9$ cells were harvested by centrifugation, resuspended in 100 µl phosphate buffered saline and lysed by heating at 95 °C for 15 min. After cooling down 0.3 µl of Benzonase (25 U μl^{-1}, Novagen) were added and the mixture was incubated at room temperature for 5 min under constant shaking. Cell lysates were mixed 1:1 with SDS loading buffer and 10 µl of each sample were separated by SDS-PAGE. Protein bands were transferred to a PVDF membrane by a semidry western blot. The membrane was blocked in 10 % (w/v) skim milk solution and incubated with a 1:1000 dilution of the primary anti-FlgZ antibody. Several washing steps were followed by incubation with the secondary goat-anti-rabbit-IgG antibody conjugated to horseradish peroxidase (Dianova) in a 1:2000 dilution. After washing, the blot was developed by using Lumi-Light Western blotting Substrate (Roche). Chemiluminescence was detected using ECL Chemocam Imager (Intas).

N-terminal protein sequencing

In order to determine the N-terminal sequence of FlgZ, a mutant carrying a *flgZ-his6* allele at its native locus was constructed. FlgZ-His6 was purified from whole cell lysate by affinity purification with Ni-NTA Agarose (Qiagen) according to manufacturer's instructions. In brief, whole cell lysate was prepared from 500 ml culture with an OD_{600} of 1.63 by sonication in lysis buffer supplemented with 1 mM DTT and cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail (Roche). Proteins of the eluate fraction were separated by SDS-PAGE, electro blotted to a PVDF membrane and stained with Ponceau S. Protein bands of the size corresponding to FlgZ-His6 (29,1 kDa) were cut out for N-terminal protein sequencing according to Edman performed on an Applied Biosystems Procise Protein Sequencer 494C with reagents supplied by the manufacturer (Life Technologies).

Swimming and swarming motility

Swimming and swarming motility assays were performed as previously described (Overhage *et al.*, 2008) with slight modifications. Briefly, swimming was performed on BM2 glucose medium containing 0.3 % agar in a 6-well plate format. Swarming was examined in petri dishes (85 mm diameter) on modified BM2 glucose plates containing 0.1 % CAS amino acids as only nitrogen source and 0.5% agar. If required 0.2 % arabinose was added to induce the p_{BAD} promoter. Bacterial pre-cultures were grown for 6 h at 37 °C in LB medium with appropriate amounts of antibiotics at 180 rpm. Cells were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS). Each swimming plate was inoculated with 2 x 10^7 cells, swarming plates were inoculated with 6.6 x 10^6 cells. The plates were incubated at 30 °C in a humid atmosphere for 15 h. Every experiment was performed in at least triplicates.

Generation of a suppressor mutant

To isolate spontaneous suppressor mutants, 4 x 10^7 bacteria that overexpressed FlgZ were inoculated into the center of a fresh swarm agar plate. After 17 h of incubation at 37 °C, the plate was rinsed with PBS to harvest bacteria from the swarm colony. Approximately 1 x 10^6 bacteria of this cell suspension were used to inoculate the next round of swarming on freshly prepared plates. This step was repeated for several days. Every round was inoculated in triplicates and the colony with the largest surface coverage was chosen for the next round. The suppressor mutants were isolated after 7 rounds and saved for further experiments.

Whole genome sequencing

Genomic DNA was isolated from cells grown over night in LB with 400 µg ml^{-1} carbenicillin using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions. Whole genome sequencing libraries were generated using the TruSeq DNA PCR-free kit (Illumina) according to manufacturer's instructions. Paired-end sequencing (2 × 250 bp) was performed on a MiSeq platform (Illumina) using MiSeq Reagent Kits v2 chemistry (Illumina). Sequencing resulted in 2.5 million reads for PAO1 overexpressing *flgZ* (sample SBE1) and 1.8 million and 1.6 million reads for the repressor mutants 1 (sample SBE2) and 2 (sample SBE3), respectively.
Reads were mapped to the genome of *P. aeruginosa* PAO1 (Stover *et al.*, 2000); first with bwa (Li and Durbin, 2009) and non-perfectly mapped reads were re-mapped with stampy (Lunter and Goodson, 2011) resulting in a coverage of 68-fold, 42-fold and 41-fold for samples SBE1, SBE2 and SBE3, respectively. Mapping qualities were analysed using picard tools (https://broadinstitute.github.io/picard/). Single nucleotide polymorphisms and short indels were identified with samtools mpileup, filtered with bcftools (Li, 2011) (Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)(Li, 2011)and annotated with SnpEff (Cingolani *et al.*, 2012). Large deletions (i.e. regions with no coverage) were identified using BEDTools (Quinlan and Hall, 2010) and manually confirmed using the Integrative Genomics Viewer (Robinson *et al.*, 2011).

The sequencing data has been deposited at the National Center for Biotechnology Information sequence read archive (http://www.ncbi.nlm.nih.gov/sra) under the BioProject Accession No. PRJNA495500.

Negative staining of flagellar filaments and electron microscopy

Thin carbon support films were prepared by sublimation of carbon on to a freshly cleaved mica surface. Bacteria were negatively stained with 2 % (w/v) aqueous uranyl acetate, pH 5.0, according to the method of Valentine et al. (Valentine *et al.*, 1968). In brief, bacteria were adsorbed onto the carbon film for 20 sec and washed in TE buffer (20 mM TRIS, 2 mM EDTA, pH 6.9), distilled water and then collected with 300 mesh copper grids. Samples were air dried under a lamp before examination in a TEM 910 transmission electron microscope (Carl Zeiss, Oberkochen) at an acceleration voltage of 80 kV. Images were taken at calibrated magnifications using a line replica. Images were recorded digitally with a Slow-Scan CCD-Camera (ProScan, 1024x1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany).

Fluorescence microscopy

Bacteria were grown in LB supplemented with gentamicin and 0.2 % arabinose to induce expression of the fusion proteins. Upon entry into early stationary phase after 5-6 h of growth, bacteria were fixed with 4 % paraformaldehyde solution for 1h at room temperature. Between 5 to 10 µl of the fixed cells were spotted onto an agar pad (phosphate buffered saline solidified with 1.5 % (w/v) agarose) which was placed into a 35 mm glass bottom µ-Dish (ibidi). Fluorescence images were recorded by a Nikon eclipse Ti microscope (Nikon) equipped with a 100 x magnification objective, the EGFP ET filter set and a Hamamatsu Orca Flash 4.0 camera by usage of the NIS elements software (Nikon). Images were analyzed with Fiji and the amount of polar localized GFP-FlgZ per image was counted manually in a blinded analysis.

Dot blot c-di-GMP binding assay

Dot blot assay were performed as previously described (Düvel *et al.*, 2012; Düvel *et al.*, 2016). Briefly, in vitro translated proteins (EasyXpress Protein Synthesis, Qiagen) were spotted on a nitrocellulose membrane. The membrane was dried, blocked with skim milk powder for 1 h at room temperature and incubated with 2'-Fluo- AHC-c-di-GMP (1 µM in TBS-T containing 5% (w/v) skimmilk powder) for 1 h at room temperature. After washing the dot blots were scanned with a FLA-9000 reader (Fujifilm) using the LPB filter and SYBR Green I settings.

Acknowledgements

The German Research Foundation (DFG) provided funding to SH in the framework of the SPP1879. The HGF impulse fund provided funding to TS under grant number W2/W3-066. We thank Christian Erck, Werner Tegge and Hildegard Schwab-Hanisch for help with the synthesis of FlgZ derived peptides and affinity purification of the anti-FlgZ antibody. We are thankful to Simon Ritter and Agata Bielecka for technical help in cloning and Illumina library preparation, to Beate Jaschok-Kentner for Edman N-terminal sequencing and to Manfred Rohde for performing transmission electron microscopy.

Author Contributions

SBe, SH and JD designed the study and wrote the manuscript. SBe and JD performed the experiments and analyzed the data. AS and TS contributed to the establishing of the microscopic analysis in the Molecular Bacteriology group. SBr analyzed the genome sequencing data and wrote this specific part of the manuscript.

References

Arnosti, D.N., and Chamberlin, M.J. (1989) Secondary sigma factor controls transcription of flagellar and chemotaxis genes in Escherichia coli. *Proc Natl Acad Sci U S A* **86**: 830–4.

Baker, A.E., Diepold, A., Kuchma, S.L., Scott, J.E., Ha, D.G., Orazi, G., *et al.* (2016) PilZ Domain Protein FlgZ Mediates Cyclic Di-GMP-Dependent Swarming Motility Control in Pseudomonas aeruginosa. *J Bacteriol* **198**: 1837–1846.

Barends, T.R.M., Hartmann, E., Griese, J.J., Beitlich, T., Kirienko, N. V., Ryjenkov, D.A., *et al.* (2009) Structure and mechanism of a bacterial light-regulated cyclic nucleotide phosphodiesterase. *Nature* **459**: 1015–1018.

Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C.A., Ackermann, M., *et al.* (2010) Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* **141**: 107–16.

Cai, Z., Liu, Y., Chen, Y., Yam, J.K.H., Chew, S.C., Chua, S.L., *et al.* (2015) RpoN Regulates Virulence Factors of Pseudomonas aeruginosa via Modulating the PqsR Quorum Sensing Regulator. *Int J Mol Sci* **16**: 28311–9.

Choi, K.-H., Kumar, A., and Schweizer, H.P. (2006) A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. *J Microbiol Methods* **64**: 391–397.

Chou, S.-H., and Galperin, M.Y. (2016) Diversity of Cyclic Di-GMP-Binding Proteins and Mechanisms. *J Bacteriol* **198**: 32–46.

Christen, M., Christen, B., Folcher, M., Schauerte, A., and Jenal, U. (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J Biol Chem* **280**: 30829–37.

Cingolani, P., Platts, A., Wang, L.L., Coon, M., Nguyen, T., Wang, L., *et al.* (2012) A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. *Fly (Austin)* **6**: 80–92.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**: 33–38.

Correa, N.E., Peng, F., and Klose, K.E. (2005) Roles of the regulatory proteins FlhF and FlhG in the Vibrio cholerae flagellar transcription hierarchy. *J Bacteriol* **187**: 6324–32.

Dasgupta, N., Arora, S.K., and Ramphal, R. (2000) fleN, a gene that regulates flagellar number in Pseudomonas aeruginosa. *J Bacteriol* **182**: 357–64.

Dasgupta, N., and Ramphal, R. (2001) Interaction of the Antiactivator FleN with the Transcriptional Activator FleQ Regulates Flagellar Number inPseudomonas aeruginosa. *J Bacteriol* **183**: 6636–6644.

Dasgupta, N., Wolfgang, M.C., Goodman, A.L., Arora, S.K., Jyot, J., Lory, S., and Ramphal, R. (2003) A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in Pseudomonas aeruginosa. *Mol Microbiol* **50**: 809–824.

Dötsch, A., Eckweiler, D., Schniederjans, M., Zimmermann, A., Jensen, V., Scharfe, M., *et al.* (2012) The Pseudomonas aeruginosa Transcriptome in Planktonic Cultures and Static Biofilms Using RNA Sequencing. *PLoS One* **7**: e31092.

Düvel, J., Bense, S., Möller, S., Bertinetti, D., Schwede, F., Morr, M., *et al.* (2016) Application of Synthetic Peptide Arrays To Uncover Cyclic Di-GMP Binding Motifs. **198**: 138–146.

Düvel, J., Bertinetti, D., Möller, S., Schwede, F., Morr, M., Wissing, J., *et al.* (2012) A chemical proteomics approach to identify c-di-GMP binding proteins in Pseudomonas aeruginosa. *J Microbiol Methods* **88**: 229–236.

Fang, X., and Gomelsky, M. (2010) A post-translational, c-di-GMP-dependent mechanism regulating flagellar motility. *Mol Microbiol* **76**: 1295–1305.

Frisk, A., Jyot, J., Arora, S.K., and Ramphal, R. (2002) Identification and Functional Characterization of flgM, a Gene Encoding the Anti-Sigma 28 Factor in Pseudomonas aeruginosa. *J Bacteriol* **184**: 1514–1521.

Gödeke, J., Heun, M., Bubendorfer, S., Paul, K., and Thormann, K.M. (2011) Roles of two Shewanella oneidensis MR-1 extracellular endonucleases. *Appl Environ Microbiol* **77**: 5342–51.

Gussin, G.N., Ronson, C.W., and Ausubel, F.M. (1986) Regulation of Nitrogen Fixation Genes. *Annu Rev Genet* **20**: 567–591.

Güvener, Z.T., and Harwood, C.S. (2007) Subcellular location characteristics of the Pseudomonas aeruginosa GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. *Mol Microbiol* **0**: 071119190133004–???

Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7**: 263–73.

Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., and Miller, S.I. (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* **436**: 1171–1175.

Ishimoto, K.S., and Lory, S. (1989) Formation of pilin in Pseudomonas aeruginosa requires the alternative sigma factor (RpoN) of RNA polymerase. *Proc Natl Acad Sci U S A* **86**: 1954–7.

Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., *et al.* (2003) Comprehensive transposon mutant library of Pseudomonas aeruginosa. .

Jenal, U., and Malone, J. (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* **40**: 385–407.

Lee, E.R., Baker, J.L., Weinberg, Z., Sudarsan, N., and Breaker, R.R. (2010) An allosteric self-splicing ribozyme triggered by a bacterial second messenger. *Science (80-)* **329**: 845–848.

493 Li, H. (2011) A statistical framework for SNP calling, mutation discovery, association mapping and
494 population genetical parameter estimation from sequencing data. *Bioinformatics* **27**: 2987–2993.

495 Li, H., and Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler
496 transform. *Bioinformatics* **25**: 1754–1760.

497 Lunter, G., and Goodson, M. (2011) Stampy: a statistical algorithm for sensitive and fast mapping of
498 Illumina sequence reads. *Genome Res* **21**: 936–9.

499 Martínez-Granero, F., Navazo, A., Barahona, E., Redondo-Nieto, M., González de Heredia, E., Baena,
500 I., *et al.* (2014) Identification of flgZ as a Flagellar Gene Encoding a PilZ Domain Protein That
501 Regulates Swimming Motility and Biofilm Formation in Pseudomonas. *PLoS One* **9**: e87608.

502 Murray, T.S., and Kazmierczak, B.I. (2006) FlhF is required for swimming and swarming in
503 Pseudomonas aeruginosa. *J Bacteriol* **188**: 6995–7004.

504 Okegbe, C., Fields, B.L., Cole, S.J., Beierschmitt, C., Morgan, C.J., Price-Whelan, A., *et al.* (2017)
505 Electron-shuttling antibiotics structure bacterial communities by modulating cellular levels of c-di-
506 GMP. *Proc Natl Acad Sci U S A* **114**: E5236–E5245.

507 Overhage, J., Bains, M., Brazas, M.D., and Hancock, R.E.W. (2008) Swarming of Pseudomonas
508 aeruginosa is a complex adaptation leading to increased production of virulence factors and
509 antibiotic resistance. *J Bacteriol* **190**: 2671–9.

510 Paul, K., Nieto, V., Carlquist, W.C., Blair, D.F., and Harshey, R.M. (2010) The c-di-GMP Binding Protein
511 YcgR Controls Flagellar Motor Direction and Speed to Affect Chemotaxis by a "Backstop Brake"
512 Mechanism. *Mol Cell* **38**: 128–139.

513 Paul, R., Weiser, S., Amiot, N.C., Chan, C., Schirmer, T., Giese, B., and Jenal, U. (2004) Cell cycle-
514 dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase
515 output domain. *Genes Dev* **18**: 715–27.

516 Pesavento, C., Becker, G., Sommerfeldt, N., Possling, A., Tschowri, N., Mehlis, A., and Hengge, R.
517 (2008) Inverse regulatory coordination of motility and curli-mediated adhesion in Escherichia coli.
518 *Genes Dev* **22**: 2434–46.

519 Quinlan, A.R., and Hall, I.M. (2010) BEDTools: a flexible suite of utilities for comparing genomic
520 features. *Bioinformatics* **26**: 841–842.

521 Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov,
522 J.P. (2011) Integrative genomics viewer. *Nat Biotechnol* **29**: 24–26.

523 Römling, U., Galperin, M.Y., and Gomelsky, M. (2013) Cyclic di-GMP: the first 25 years of a universal
524 bacterial second messenger. *Microbiol Mol Biol Rev* **77**: 1–52.

525 Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., *et al.* (1987)
526 Regulation of cellulose synthesis in Acetobacter xylinum by cyclic diguanylic acid. *Nature* **325**: 279–
527 281.

528 Rossmann, F., Brenzinger, S., Knauer, C., Dörrich, A.K., Bubendorfer, S., Ruppert, U., *et al.* (2015) The
529 role of FlhF and HubP as polar landmark proteins in *S hewanella putrefaciens* CN-32. *Mol Microbiol*
530 **98**: 727–742.

531 Ryan, R.P., Fouhy, Y., Lucey, J.F., Crossman, L.C., Spiro, S., He, Y.-W., *et al.* (2006) Cell-cell signaling in
532 Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic di-GMP
533 turnover. *Proc Natl Acad Sci U S A* **103**: 6712–7.

Ryjenkov, D.A., Tarutina, M., Moskvin, O. V, and Gomelsky, M. (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J Bacteriol* **187**: 1792–8.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: a laboratory manual. *Mol cloning a Lab manual* .

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., *et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**: 676–682.

Schmidt, A.J., Ryjenkov, D.A., and Gomelsky, M. (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J Bacteriol* **187**: 4774–81.

Schulz, S., Eckweiler, D., Bielecka, A., Nicolai, T., Franke, R., Dötsch, A., *et al.* (2015) Elucidation of Sigma Factor-Associated Networks in Pseudomonas aeruginosa Reveals a Modular Architecture with Limited and Function-Specific Crosstalk. *PLOS Pathog* **11**: e1004744.

Semmler, A.B.T., Whitchurch, C.B., Leech, A.J., Mattick, J.S., and Semmler, A.B.T. (2000) Identification of a novel gene, fimV, involved in twitching motility in Pseudomonas aeruginosa. *Microbiology* **146**: 1321–1332.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., *et al.* (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **7**: 539.

Starnbach, M.N., and Lory, S. (1992) The filA (rpoF) gene of Pseudomonas aeruginosa encodes an alternative sigma factor required for flagellin synthesis. *Mol Microbiol* **6**: 459–469.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., *et al.* (2000) Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature* **406**: 959–964.

Sudarsan, N., Lee, E.R., Weinberg, Z., Moy, R.H., Kim, J.N., Link, K.H., and Breaker, R.R. (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* **321**: 411–413.

Tarutina, M., Ryjenkov, D.A., and Gomelsky, M. (2006) An unorthodox bacteriophytochrome from Rhodobacter sphaeroides involved in turnover of the second messenger c-di-GMP. *J Biol Chem* **281**: 34751–8.

Totten, P.A., Lara, J.C., and Lory, S. (1990) The rpoN gene product of Pseudomonas aeruginosa is required for expression of diverse genes, including the flagellin gene. *J Bacteriol* **172**: 389–96.

Tuckerman, J.R., Gonzalez, G., Sousa, E.H.S., Wan, X., Saito, J.A., Alam, M., and Gilles-Gonzalez, M.-A. (2009) An Oxygen-Sensing Diguanylate Cyclase and Phosphodiesterase Couple for c-di-GMP Control. *Biochemistry* **48**: 9764–9774.

Valentine, R.C., Shapiro, B.M., and Stadtman, E.R. (1968) Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from Escherichia coli. *Biochemistry* **7**: 2143–2152.

Valentini, M., and Filloux, A. (2016) Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from *Pseudomonas aeruginosa* and Other Bacteria. *J Biol Chem* **291**: 12547–12555.

Wehbi, H., Portillo, E., Harvey, H., Shimkoff, A.E., Scheurwater, E.M., Howell, P.L., and Burrows, L.L. (2011) The peptidoglycan-binding protein FimV promotes assembly of the Pseudomonas aeruginosa type IV pilus secretin. *J Bacteriol* **193**: 540–50.

Winsor, G.L., Griffiths, E.J., Lo, R., Dhillon, B.K., Shay, J.A., and Brinkman, F.S.L. (2016) Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the Pseudomonas genome database. *Nucleic Acids Res* **44**: D646–D653.

Wirebrand, L., Österberg, S., López-Sánchez, A., Govantes, F., and Shingler, V. (2018) PP4397/FlgZ provides the link between PP2258 c-di-GMP signalling and altered motility in Pseudomonas putida. *Sci Rep* **8**: 12205.

Wurtzel, O., Yoder-Himes, D.R., Han, K., Dandekar, A.A., Edelheit, S., Greenberg, E.P., *et al.* (2012) The Single-Nucleotide Resolution Transcriptome of Pseudomonas aeruginosa Grown in Body Temperature. *PLoS Pathog* **8**: e1002945.

Yamaichi, Y., Bruckner, R., Ringgaard, S., Möll, A., Cameron, D.E., Briegel, A., *et al.* (2012) A multidomain hub anchors the chromosome segregation and chemotactic machinery to the bacterial pole. *Genes Dev* **26**: 2348–60.

Figure legends

Figure 1: Growth phase dependent transcription and translation of *flgZ*

A FlgZ protein levels of *P. aeruginosa* PAO1 grown in exponential and stationary growth phase (adjusted to the same optical density) were determined by Western blot analysis using an anti-FlgZ antibody.

B Relative fold change of *flgM, flgN* and *flgZ* gene expression in stationary growth phase compared to exponential growth phase. Data were extracted from a previous publication (Dötsch *et al.*, 2012). RNA sequencing was performed with cultures grown to OD 1 (exponential) and for 12 h (stationary). Both were sequenced in duplicates. The obtained sequences were mapped to the PA14 genome. Absolute read counts were normalized to yield nRPK (normalized reads per kilobase of gene sequence) values.

C A set of six primers was used in different combinations to PCR-amplify intragenic (1, 2, 3) and intergenic (4, 5) regions of the *flgMNZ* genes as indicated.

D Reverse Transcriptase PCR: cDNA was generated from RNA isolated from exponential (left) and from early stationary (right) growth phase. Lane 1-3 correspond to the intragenic PCR products of *flgM* (1), *flgN* (2), *flgZ* (3), lane 4-5 to the intergenic *flgMN* (4) and *flgNZ* (5) PCR products. As negative control (NC) total RNA was not subjected to reverse transcription.

Figure 2: **Schematic presentation of the *flgMNZ* operon.** The *flgZ* specific transcriptional start site (TSS 3) identified by 5´RACE is displayed in orange. This TSS 3 is located within the annotated *flgZ* gene at +17 bp. The genomic sequence information of *flgZ* (framed in red) depicts a putative alternative start codon (GTG) as well as an alternative ribosomal binding site (RBS 2).

Figure 3: Re-annotation of the *flgZ* open reading frame.

A Multiple-sequence alignment of the *flgZ* gene start in different *Pseudomonas* species generated with the Clustal Omega Software (Sievers *et al.*, 2011). Sequences were obtained from the Pseudomonas Genome Database (Winsor *et al.*, 2016). The *flgZ* gene sequences of the two *P. aeruginosa* strains PAO1 and PA14 were aligned with orthologues of *P. putida* KT2440 (Pp *flgZ*), *P. fluorescens* F113 (Pf *flgZ*), *P. protegens* Pf-5 (PFL_4486), *P. syringae pv. tomato* DC3000 (PSPTO_1923), *P. stutzeri* A1501 (PST_1382) and *P. mendocina* ymp (Pmen_2856). The annotated start codon is shown in green, the alternative start of *flgZ* in *P. aeruginosa* is shown in green and is underlined. The ribosomal binding sites are depicted in blue.

B N-terminal sequencing revealed the red framed amino acids (PNPFVEEAGP) as the N-terminus of FlgZ. The annotated and the re-annotated start codons are depicted in green, the identified TSS is shown in orange and the RBS in blue.

Figure 4: Analysis of a *flgZ* specific promoter and its transcriptional regulation by sigma factors

A Information on log2 fold changes of *flgM, flgN* and *flgZ* gene expression in sigma factor knock out (*ΔfliA*, *ΔrpoN* and *ΔrpoS*) and overexpressing strains (oe) relative to wild type and wild type containing the empty vector control, respectively, were extracted from a previous publication (Schulz *et al.*, 2015). A significant negative regulation is marked in red and a significant positive regulation is marked in green (p-value < 0.05).

B Activity of the transcriptional fusion of the *flgZ* promoter region with *luxCDABE* genes in the PA14 wild type and sigma factor knock out mutants *ΔfliA*, *ΔrpoN* and *ΔrpoS* in exponential (exp) and stationary (stat) growth phase. The obtained luminescence signal was normalized to the optical density and the empty vector control.

Figure 5: Motility behavior of *flgZ* mutant and *flgZ* overexpressing strains.

A C-di-GMP binding assays were performed with *in vitro* synthesized FlgZ proteins containing either the native RNAYR motif or the mutated ANAYA motif. Proteins were spotted on a membrane and incubated with fluorescence-labeled c-di-GMP.

B Swimming motility of the wild type (wt) and the *flgZ* transposon mutant (*flgZ* tn) containing an empty vector (EV) and two *flgZ* overexpressing strains producing either the native protein (*flgZ* RNAYR) or the protein variant with the mutated c-di-GMP binding motif (*flgZ* ANAYA). Swimming agar plates were incubated for 16 h at 30 °C in a humid atmosphere. Diameters of swimming zones were measured in three independent experiments of three replicates each. Significance was determined by analysis of variance and a Bonferroni´s posttest, * $p \leq 0.05$, *** $p \leq 0.0001$.

C Swarming motility of the indicated strains after 16 h of incubation at 30 °C in humid atmosphere. One representative swarming plate out of three independent experiments with three technical replicates is shown.

Figure 6: ***ΔfimV* counteracted the negative effect of FlgZ on motility**

A Generation of a suppressor mutant by an extended swarming motility assay. Bacterial cells of the wild type containing the empty vector (wt EV) and the *flgZ* overexpressing strain (*flgZ* tn *flgZ* oe) were transferred to a freshly prepared swarming agar plate on a daily basis. Plates were incubated at 30 °C in humid atmosphere for 16 h. After seven days we selected a swarming colony of wild type size despite *flgZ* overexpression. Whole genome sequencing revealed a partial deletion in *fimV* generating the respective phenotype.

B Phenotype of the suppressor mutant was controlled with an overexpression (oe) of *flgZ* in a complete *fimV* knock out mutant (*ΔfimV*) and a mutant in which the partial deletion within *fimV* was constructed (*fimV*_truncated). Swarming plates were incubated at 30 °C in humid atmosphere for 16 h. Experiments were performed in independent duplicates with three replicates each.

C Swimming motility of the wild type (wt) and the complete *fimV* knock out mutant (*ΔfimV*) containing either the empty vector (EV) or the *flgZ* overexpression vectors with the native c-di-GMP binding motif (*flgZ* RNAYR) or the mutated c-di-GMP binding motif (*flgZ* ANAYA). Swimming agar plates were incubated 16 h at 30 °C in a humid atmosphere. Diameter of the swimming zone was measured in three independent experiments of three replicates each. Significance was determined by analysis of variance and a Bonferroni´s posttest, *** $p \leq 0.0001$.

D Swarming motility of the indicated strains after 16 h of incubation at 30 °C in humid atmosphere. Plate coverage of at least seven swarming colonies originating from three independent experiments was measured with Fiji (Schindelin *et al.*, 2012). Significance was determined by analysis of variance and a Bonferroni´s posttest, ns non-significant, *** p≤ 0.0001.

Figure 7: Decreased amount of polar localized GFP-FlgZ in the *fimV* deletion mutant

A Analysis of flagella number and localization in the wild type (wt) and the *ΔfimV* mutant by transmission electron microscopy (TEM). One representative cell is shown. Scale bar=1 µm.

B Localization of GFP-FlgZ RNAYR and GFP-FlgZ ANAYA in the wild type (wt) and the *ΔfimV* mutant. One representative image per strain is shown. Scale bar 2 µm.

C Quantifications of the percentage of polar puncta in the wild type (wt) and the *ΔfimV* mutant. Fluorescent polar puncta were counted in 32 different frames of five independent experiments. Every dot represents one frame. For each strain, more than 6000 bacterial cells were analyzed and mean +/- SD are displayed. Significance was determined by performing a Kruskal-Wallis-Test and a Dunn's Multiple Comparison Test, ns non-significant, * p ≤ 0.05, *** p≤ 0.0001.