Molecular genetic and genomic analyses of the dual-function transcription regulator PdeL in *E. coli*

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Abstract

PdeL is a transcription regulator and one of 13 phosphodiesterases in *Escherichia coli* K-12. Phosphodiesterases hydrolyze the ubiquitous bacterial second messenger cyclic-di-GMP and contribute to its signaling involving bacterial lifestyle and other processes. PdeL is characterized by the presence of an N-terminal FixJ/NarL-type DNA-binding domain linked to a catalytically active, cyclic-di-GMP specific, EAL-type phosphodiesterase (PDE) domain. PdeL has been shown to positively autoregulate its own expression. No further loci have yet been shown to be regulated by PdeL.

In this work, I characterized PdeL as a transcriptional regulator of different loci, including the flagellar class 2 operon *fliFGHIJK* and *sslE*, encoding an extracellular metalloprotease. Repression of *fliFGHIJK* operon inhibits the expression of flagellar subunits needed in the early phase of flagella synthesis. As a result of repression of transcription by PdeL motility is inhibited. The DNA-binding site of PdeL at the regulatory region of *fliFGHIJK* overlaps with DNA-binding sites of flagellar master regulator $FlhD_4C_2$ and transcription regulator CsgD indicating that PdeL could be repressing *fliFGHIJK* transcription by inhibiting its activation. The expression of *sslE* is activated by PdeL. The metalloprotease SslE is important for infection of epithelial cells by *E. coli*. Regulation of *sslE* by PdeL indicates a role of PdeL in virulence. In addition, I describe a finding that overexpression of PdeL and other transcription regulators cause changes in nucleoid structure and affect cell division presumably by unspecific binding throughout the nucleoid. This finding is of particular relevance for the characterization of transcription regulators when physiological conditions under which they are expressed and active are yet poorly understood and plasmidic expression systems are employed.

1. Introduction

Environmental signals such as changes in temperature, pH and nutrient availability present a challenging task for bacteria, such as the gram-negative *Escherichia coli*. In common *E. coli* inhabits gastrointestinal areas where it can be present as a commensal bacterium or a pathogen (Croxen et al., 2013). The environment changes during host colonization, and thus the bacteria are subjected to changes in the availability of nutrients, as well as sudden shifts in pH and temperature. When these shifts occur, *E. coli* is facing different perturbations that must be overcome. The process bacteria utilize to respond and adapt to changes of available carbon sources is called stress response (Gottesman, 2019). The full process of a stress response includes sensing a stress, hence acting as an input, and then respond to it by creating an output by the bacteria. In parallel to responding to stresses, bacteria also must maintain the essential cellular machinery required throughout its life cycle. Maintenance of the essential machinery and responding to different stresses is coordinated on all levels from transcription regulation to protein activity. In this thesis, I characterized a dual function transcription regulator, PdeL. Therefore, in the following sections I will provide an overview on different cellular processes and properties such as transcription regulation, nucleoid organization, cyclic-di-GMP dependent signaling, and cell division all differently aiding in adaptation of the bacteria to environmental stimuli.

1.1. Transcription regulation

As part of a particular stress response, *E. coli* activate expression of genes specific for the individual stress sensed. The understanding of stress responses in bacteria was aided by the clarification of the mechanism for initiation of gene expression and the principles of transcription regulation (Browning and Busby, 2016; Busby, 2019; Lee et al., 2012). The expression of genes is in many cases controlled at the level of transcription initiation by RNA polymerase. Upon association of the RNA polymerase with a sigma factor, RNA polymerase binds specific promoters for transcription initiation (Helmann, 2019; Mejia-Almonte et al., 2020). By managing different sigma factors, *E. coli* can regulate the transcription of different sets of genes. The expression of essential genes, house-keeping genes and many other genes depends on the sigma factor σ^{70},

whereas alternative sigma factors are utilized for specific sets of genes that are expressed in response to specific stimuli (Busby, 2019). Further fine-tuning of transcription regulation is enabled by additional DNA-binding proteins including transcription regulators and nucleoid-associated proteins. Transcription regulators act as activators or repressors of gene expression by interacting with RNA polymerase or preventing access of RNA-polymerase to the promoter (Busby, 2019). Nucleoid-associated proteins (NAPs) are usually abundant and bind at many sites on the nucleoid and contribute to transcription regulation (Dillon and Dorman, 2010).

1.2. Nucleoid associated proteins and transcription regulators

In *E. coli*, different DNA-binding proteins are present such as nucleoid-associated proteins (NAPs) and transcription regulators.

Nucleoid-associated proteins are abundant and bind at many sites of the nucleoid with low specificity. Nucleoid associated proteins have been shown to control nucleoid organization and regulate gene expression by abundant binding to the nucleoid. One such nucleoid associated protein is the histone-like nucleoid structuring protein H-NS involved in silencing of horizontally acquired genes (Navarre et al., 2006). In brief, H-NS binds preferentially to AT-rich sequences and thus can interfere with binding of RNA-polymerase at the promoter region of genes, which are AT-rich (Grainger, 2016). Further, a positive correlation between AT-content of horizontally acquired genes and repression by H-NS was shown (Lucchini et al., 2006). Repression by H-NS of approximately 5% of all genes in the *E. coli* chromosome, many of which are horizontally acquired genes allowed classification of H-NS as a xenogenic silencer (Hommais et al., 2001; Navarre, 2016). A role of H-NS in structuring of the chromosome has been discussed and is subject of further analysis (Lioy et al., 2018). Other nucleoid-associated proteins are also present in *E. coli.* Depending on their DNA-binding properties, nucleoid-associated proteins contribute to nucleoid organization as well as regulation of gene expression (Verma et al., 2019). The histone-like protein HU has been shown to be important for condensation of the nucleoid, but also to be functioning as a co-factor in the repression of the *gal* operon (Aki et al., 1996; Hammel et al., 2016). Functional overlap between nucleoid organization and regulation of gene expression

depending on specificity of DNA-binding and protein abundance makes a clear separation of transcription regulators and nucleoid-associated proteins questionable (Dorman et al., 2020).

As part of this thesis, a possible influence of the abundance of specific transcription regulators on nucleoid organization has been identified. The finding that transcription regulators binding DNA affects nucleoid organization further supports the questionable separation of nucleoid associated proteins and transcription regulators.

Transcription regulators specifically bind to promoter regions of genes and alter binding of the RNA polymerase holoenzyme to the promoter usually by interaction or inhibiting binding of RNA polymerase for initiation of transcription. Upon binding of a transcription activator, the RNA polymerase holoenzyme is in many cases recruited to the promoter where it binds to specific sequence motifs, such as the -35 and -10 elements in case of σ^{70} (Busby, 2019). A classic example for regulation of transcription is the regulation of the *lac* operon by the transcription repressor LacI and the transcription activator CRP (Lewis, 2011). In brief, LacI binds to the *lac* operator sequence and inhibits transcription initiation. Upon binding of the physiological effector allolactose to LacI, a structural change of LacI is induced which lowers affinity of LacI to the *lac* operator (Lewis, 2005). The transcription activator CRP undergoes structural changes and is activated upon binding of cAMP produced at low glucose levels. Activated CRP can then bind to the operator sequence and enhance transcription initiation (Majors, 1975; Zubay et al., 1970). Transcription regulators form a complex regulatory network controlling gene expression. While the RNA polymerase holoenzyme with associated sigma factor is capable to specifically bind promoters and initiate transcription, approximately 300 transcription regulators fine-tune expression of genes. Transcription regulators have been analyzed for their ability to bind DNA and grouped according to structural and sequence similarities (Ishihama et al., 2016). The DNA sequence preferentially bound by an individual transcription regulator can be used to describe a DNA-binding motif, and is one of the criteria used to create families of transcription regulators with similar properties as described in the next section.

1.3. Families of transcription regulators

Transcription regulators have been classified into several families based on their various properties including sequence, topology and functionality (Ishihama et al., 2016). Further, publications on transcription regulators of *E. coli* are curated in the RegulonDB database and used to compute regulatory networks (Santos-Zavaleta et al., 2019). Approximately 300 transcription regulator are presumably present in *E. coli* (Perez-Rueda and Collado-Vides, 2000). One family of transcription regulators is characterized by their FixJ/NarL-type DNA-binding domain. FixJ/NarL-type DNA-binding proteins make up a significant portion of response regulators in two-component systems for stress response in the bacterial phyla (Galperin, 2010). Response regulators of two-component systems characteristically encompass an N-terminal receiver domain, used for input of environmental signals, linked to a C-terminal output domain such as a DNA-binding domain. One well studied member of the FixJ/NarL-type DNA-binding protein family is the transcription regulator RcsB involved in activation of capsule synthesis upon cell surface perturbation (Wall et al., 2018). One atypical member of the FixJ/NarL-type DNA-binding protein family is the cylic-di-GMP phosphodiesterase PdeL. PdeL encompasses an N-terminal DNA-binding domain linked to a C-terminal phosphodiesterase domain. The presence of a DNA-binding domain linked to a phosphodiesterase domain is a rare feature within transcription regulators and cyclic-di-GMP effector proteins alike. PdeL's ability to autoregulate its gene expression and its enzymatic function as one of 13 phosphodiesterases in *E. coli* has led to its proposed classification as a trigger enzymes in the context of cyclic-di-GMP signaling (Hengge, 2016). Trigger enzymes are defined as proteins whose enzymatic activity is used as sensor to generate signal outputs such as regulation of gene expression, which in case of PdeL is in the context of cyclic-di-GMP signaling, as described in the next section (Hengge, 2016).

1.4. Cyclic-di-GMP signaling and the bacterial lifestyle

The ubiquitous second messenger nucleotide cyclic-di-GMP plays a role in signaling in bacteria (Hengge et al., 2019). Second messenger signaling adds an additional system for bacterial signal transduction. Cellular levels of cyclic-di-GMP are controlled on the level of its synthesis and hydrolysis. In *E. coli* K-12 29 enzymes are present that are involved in synthesizing (diguanylate cyclases, DGC) or hydrolyzing (phosphodiesterases, PDE) cyclic-di-GMP (Hengge et al., 2016). Two GTP molecules are required for synthesis of cyclic-di-GMP by DGCs. Hydrolysis of cyclic-di-GMP to two GMP molecules is catalyzed by EAL/HD-GYP domains of PDEs (Römling et al., 2013). This redundancy in enzymes with their individual domain structures and expression patterns could function as a gateway for cyclic-di-GMP signaling upon sensing. Sensing of cyclic-di-GMP requires effectors. These effectors can be proteins as well as RNA riboswitches (Römling et al., 2013). Binding of cyclic-di-GMP to proteins and RNAs allows for signal transduction on a transcriptional, post-transcriptional and post-translational level depending on the cellular level of cyclic-di-GMP. Sigma factor RpoS-dependent gene expression of at least five proteins involved in the synthesis and hydrolysis of cyclic-di-GMP adds complexity to the regulation of cellular cyclic-di-GMP levels and supports a role of cyclic-di-GMP in stress response (Weber et al., 2006). Cyclic-di-GMP has been shown to be important for lifestyle transition between a motile and sessile state (D'Argenio and Miller, 2004; Römling and Galperin, 2017; Römling et al., 2005). In general, motility is controlled at a transcriptional, post-transcriptional and protein level. The housekeeping sigma factor σ^{70} initiates transcription of the flagellar master regulator FlhDC, responsible for activating expression of genes required for flagella synthesis (Fitzgerald et al., 2014). Cyclic-di-GMP is involved at a post-transcriptional and protein level in bacterial lifestyle regulation. The cyclic-di-GMP effector protein and cellulose synthase BcsA, required for formation of biofilm was shown to be activated upon binding of cyclic-di-GMP (Ross et al., 1987; Steiner et al., 2013; Weinhouse et al., 1997). In addition, cyclic-di-GMP mediates transition in lifestyle by interaction with motor brake protein YcgR to control speed and direction of the flagellar rotation in *E. coli* (Boehm et al., 2010; Fang and Gomelsky, 2010; Paul et al., 2010).

Virulence as a target for cyclic-di-GMP has also been shown. Horizontal gene transfer of a *dgc* gene into a pathogenic *E. coli* strain caused a hike in haemolytic uraemic syndrome (HUS)

developing patients in Germany, partially due to the increased synthesis of cyclic-di-GMP (Richter et al., 2014). The finding that increased cyclic-di-GMP synthesis lead to increased virulence further supports the hypothesis that fine-tuning of signal transduction is achieved by dynamic control of the intracellular level of cyclic-di-GMP with the help of diguanylatecyclases and phosphodiesterases.

PdeL is one of 13 phosphodiesterases in *E. coli*. PdeL is unique in its domain topology as it is the only PDE in *E. coli* with an N-terminal DNA-binding domain suggesting a dual functionality. In case of PdeL it is an open question whether the ability to associate cyclic-di-GMP plays a role for PdeL DNA-binding and transcription regulatory function. As part of this work, I identified PdeL as a transcription regulator repressing the expression of the *fliFGHJIK* operon. The *fliFGHIJK* operon encodes for subunits of the flagellar basal body (Altegoer et al., 2014).

In brief, the flagellum consists of multiple subunits including the basal body, the hook, the hook-filament junction and the filament. The temporal sequence in which the subunits are synthesized and assembled is tightly controlled (summarized in Fitzgerald et al, 2014). The master regulator of flagellar synthesis in *E. coli* is the transcription regulator FlhDC. The flagellar transcription network is divided into three classes dependent on the regulation of the respective promoters (Fig. 1A). The *flhDC* operon itself is considered class 1. All genes regulated by the master regulator FlhDC and transcribed by the standard RNA polymerase Sigma 70 holo-enzyme are considered class 2. The gene encoding for alternative sigma factor FliA (Sigma 28) is also a class 2 gene. Genes transcribed by the RNA polymerase Sigma 28 holoenzyme are class 3 genes. Genes regulated by FlhDC and transcribed by RNA polymerase Sigma 70 or Sigma 28 holoenzyme are classified as class 2/3 genes. This cascade of differently regulated genes enables expression of genes in dependence to the stage of flagellar synthesis (Fig. 1B) (Fitzgerald et al., 2014).

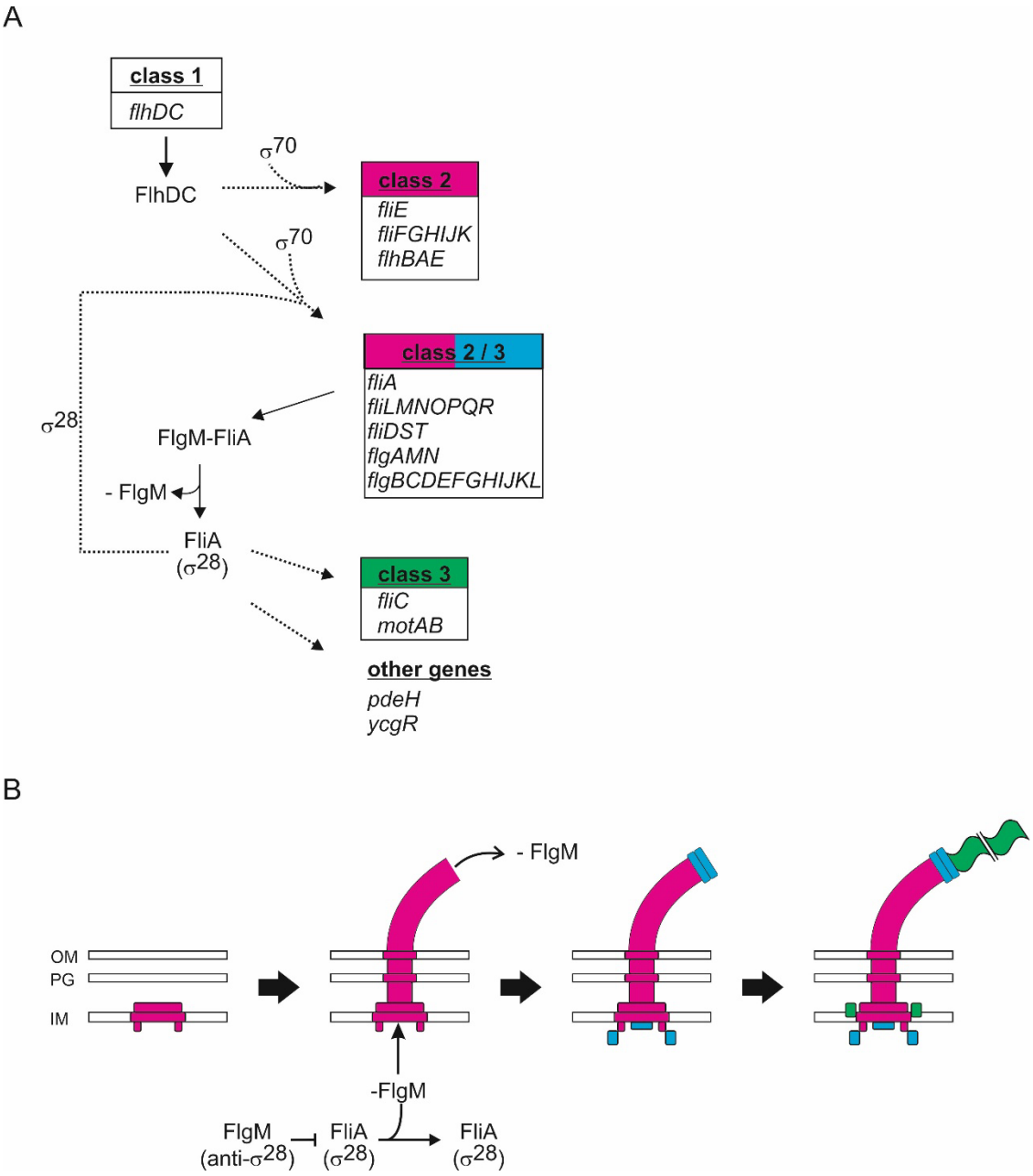

Figure 1: Simplified scheme of the flagellar transcription network controlled by the master regulator FlhDC adopted from Fitzgerald et al., (2014) and Altegoer et al., (2014). (A) The transcription network is divided into hierarchical classes. Class 1 is composed of *flhDC* encoding for the master regulator FlhDC. FlhDC regulates expression of class 2 genes which are transcribed by the RNA-polymerase Sigma 70 holoenzyme. Class 2 operons encompass genes coding for subunits of the flagellum required during early phase for basal body and export apparatus assembly. Alternative sigma factor FliA (σ^{28}) and its anti-sigma factor FlgM are also encoded by class 2 genes. FliA (σ^{28}) is bound by anti-sigma factor FlgM and thus inhibited from interacting with the RNA-polymerase for initiation of σ^{28} dependent transcription. After assembly of the export apparatus the anti-sigma factor FlgM is

exported out of the cell and FliA is released (Fitzgerald et al., 2014). Genes regulated by FlhDC and transcribed by RNA-polymerase Sigma 70 or Sigma 28 holenzymes are classified as class 2/3. In addition, RNA-polymerase Sigma 28 holoenzyme can initiates transcription of class 3 genes. (B) Scheme depicting the process of flagellar assembly. Subunits are colored according to the class of genes they are encoded by as shown in (A). OM, outermembrane; PG, peptidoglycan;IM , inner membrane.

The placement and number of synthesized flagella in bacteria, of which five to six are present in *E. coli*, is not yet clearly understood (Schuhmacher et al., 2015). By utilizing the surface exposed flagella, *E. coli* can move through semi-solid and liquid media by either swimming or swarming (Kearns, 2010). The current understanding is that a motile lifestyle of bacteria in comparison to a sessile one is driven by the decision of the bacteria to either grow or survive (Hengge, 2020). While movement through the environment is beneficial for finding new nutrients, formation of a biofilm can be beneficial for surviving external stress factors such as antimicrobial agents and other stress factors (Hengge, 2020). By formation of a biofilm, a very small proportion of bacteria can become dormant persisters, responsible for repopulating biofilms after an antimicrobial treatment of an infectious disease (reviewed in Lewis, 2007).

The regulatory processes of transcription regulation and second messenger signaling described in this and previous sections describe some of the mechanisms *E. coli* utilizes to respond and adapt to environmental stimuli on a transcriptional level. DNA-binding proteins such as transcription regulators have to be able to associate and interact with the nucleoid. Structuring of the nucleoid is hence of utter importance considering the spatial limitation within the bacterial cell. A bacterial chromosome is approximately >1 mm in length but condensed to an area of <1 µM in diameter within the bacteria (Wang et al., 2013). Therefore, condensation of the nucleoid by approximately 1000-fold allows it to fit into the bacterium (Wang et al., 2013). Coordination of transcription regulation and other processes such as replication and cell division are achieved by organization and structural maintenance of the chromosome as described in the next section.

1.5. Structural maintenance of the chromosome

Coordination between topological organization of the nucleoid and cellular processes such as DNA replication and cell division allow proliferation of bacteria (Wang et al., 2013). In this thesis I found by serendipity that overexpression of PdeL and other transcription regulators affects the nucleoid structure. Therefore, in the following section key steps in nucleoid organization and chromosome segregation are summarized.

Screens for anucleate cells have identified proteins involved in chromosome partioning (Hiraga et al., 1989). The absence of MukB led to formation of anucleate cells (Hiraga et al., 1991). MukB is a component of the bacterial structural maintenance of chromosome (SMC) complex, named MukBEF in *E. coli* (Fig. 2). Two processes which MukBEF is important for are chromosome segregation and nucleoid organization. MukBEF has been shown to organize the structure of the nucleoid by formation of an axial core for loop extrusion (Makela and Sherratt, 2020)(Fig. 2). Weakly elevated levels of a fluorescent MukB fusion indicated occupation of large regions of the nucleoid with an excluded area in the *ter* region from where MukB is displaced by MatP (Makela and Sherratt, 2020). Further, MukB mediates segregation of sister chromatids by recruitment of Topoisomerase IV during replication (Fig.2)(Nolivos et al., 2016; Zawadzki et al., 2015).

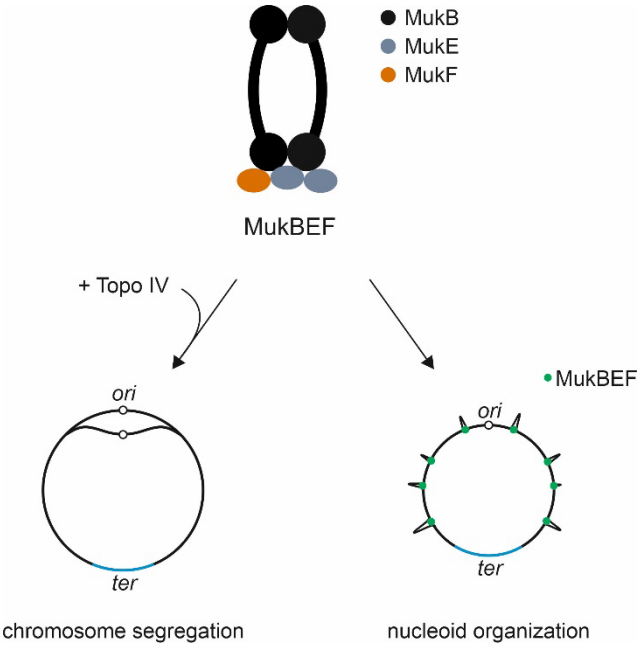

Figure 3: Scheme depicting structure and function of structural maintenance of chromosome complex MukBEF in *E. coli,* adapted from Makela et al., (2020). The MukBEF complex is composed of a MukB homodimer, two MukE and one MukB protein. MukBEF is the bacterial SMC complex bound to the nucleoid via a MukB dimer. Dimerization of MukB is dependent on its interaction with MukE and MukF. DNA-binding of MukBEF has been shown to form extrusion loops of 30-50kb DNA important for organization of the nucleoid (Makela and Sherratt, 2020). Formation of loops occurs in all regions of the chromosome except for the *ter* region where MatP mediates dissociation of MukB from the nucleoid (Nolivos et al., 2016). MukBEF further mediates the segregation of decatenated DNA by topoisomerase IV during replication (Zawadzki et al., 2015).

Although the coordination of replication initiation and cell division are still subject of research and modeling (Kleckner et al., 2018; Zaritsky and Woldringh, 2015), mechanisms that regulate cell division dependent on nucleoid organization have been identified (Hajduk et al., 2016). Two important systems are the nucleoid occlusion and the Min system which coordinate the formation of the Z-ring at the correct position and condition of the bacteria as described in the next section.

1.6. Cell division in *E. coli*

Positioning of the Z-ring in areas other than the cell division site can be fatal for the fate of the bacteria. Septation of the bacteria can hypothetically shear the nucleoid or give rise to anucleate cells. Several regulatory systems that ensure the positioning and timing of the Z-ring formation during the bacterial life cycle are summarized below.

At the cell division site FtsZ molecules form filaments by polymerization and mediate formation of the Z-ring. The nucleoid occlusion system ensures that no Z-ring can be formed while the nucleoid is in proximity of the division site. Nucleoid occlusion is driven by the action of SlmA protein which inhibits Z-ring formation in areas where the nucleoid is present (Bernhardt and de Boer, 2005). The cell division inhibitor protein SlmA binds to SlmA binding sequences (SBS) on the chromosome (Cho et al., 2011). SlmA binding sites are distributed across the chromosome with the exception of the *ter* region (Tonthat et al., 2011). Binding of SlmA to DNA activates SlmA for interaction with FtsZ. Interaction of activated SlmA with FtsZ antagonizes FtsZ polymerization and thus creates a nucleoid occlusion zone where no Z-ring can be formed (Cho et al., 2011; Tonthat et al., 2013).

The second regulatory system controlling positioning of the Z-ring is the Min system. The Min system utilizes the spatially oscillating proteins MinCDE to inhibit formation of the Z-ring near the cell poles (reviewed in Rowlett and Margolin, 2015).

In this work, I showed by serendipity that overexpression of PdeL led to defects in cell division. This finding raised the question whether PdeL could be involved in regulating cell division. PdeL could theoretically regulate cell division by transcriptional regulation of multiple genes, including the negative regulatory systems of nucleoid occlusion and Min system as well as genes essential for the cell division machinery. Therefore, a general understanding on PdeL, its transcription regulatory function and enzymatic activity is summarized in the next section.

1.7. The transcription regulator and cyclic-di-GMP phosphodiesterase PdeL

The dual function transcription regulator and cyclic-di-GMP phosphodiesterase PdeL was one of the first PDEs analyzed for its enzymatically active PDE-domain (Schmidt et al., 2005). Functional conservation of PdeL is further supported by more than 90% sequence identity within 200 *E. coli* species, including enteropathogenic EHEC strains (UniProt, 2021). Schmidt et al. (2005) set the basis for understanding the activity of PDE domains, in particular for the $PdeL_{EAL}$ domain which is enzymatically active independent of the presence of the DNA-binding domain. The structural properties of the $PdeL_{EAL}$ domain showed that binding of the substrate cyclic-di-GMP to the PDE-domain enhances dimerization (Sundriyal et al., 2014). This substrate binding enhanced dimerization of the $PdeL_{EAL}$ domain has led to the hypothesis that PdeL could function as either a DNA-binding dependent PDE or cyclic-di-GMP binding dependent transcription regulator (Sundriyal et al., 2014). In addition, screens for suppressors of a motility defect of the *pdeH* mutant, encoding the main PDE in *E. coli*, yielded *pdeL* variants (Reinders et al., 2016). The deletion of the main phosphodiesterase *pdeH* leads to elevated levels of cyclic-di-GMP which, which results in inhibition of motility by the c-di-GMP-dependent motor brake protein YcgR (Boehm et al., 2010; Fang and Gomelsky, 2010; Paul et al., 2010). Additional analysis revealed that the identified mutants of *pdeL* had increased expression and the mutant protein a putatively higher enzymatic activity which led to restored low intracellular levels of cyclic-di-GMP permissive for motility (Reinders et al., 2016). Reinders et al. (2016) phrased a model that increased protein levels of the *pdeL* mutants were due to increased positive autoregulation. These findings support the idea of PdeL as a functional transcription regulator.

1.8. Aims of this thesis

The aim of this thesis, encompassing one published article (Yilmaz et al., 2020) and two manuscripts, was an in-depth characterization of the unique PdeL as a transcription regulator in *E. coli.* Approaches employed to systematically characterize PdeL, started from expression analysis of *pdeL* itself, followed by the characterization of PdeL´s function as a transcription regulator, and analysis of the cellular localization of PdeL.

During my bachelor and master thesis on PdeL I initiated the analysis of *pdeL* regulation. Key to analyzing the cellular function of PdeL and transcription regulators in general, is understanding under which physiological conditions the transcription regulator is expressed and activated. To this end, to analyze *pdeL* expression expression studies, immunodetection, and fluorescence microscopy were employed. In brief, I analyzed *pdeL* expression in dependence of the growth phase, as well as it's regulation by global regulators and autoregulation. In addition, I tested *pdeL* expression at varied cellular cyclic-di-GMP levels. These experiments revealed that *pdeL* expression is very low.

After having established a general understanding of *pdeL* expression, I analyzed PdeL's transcription regulatory function. Therefore, I performed a RNA-sequencing analysis, which revealed differentially expressed genes in *E. coli* with elevated levels of PdeL provided from a plasmid. Putative specific target genes of PdeL were analyzed and validated by different experimental approaches such as expression analysis, flow cytometry and *in vitro* DNA-binding assays. In addition, phenotypic analyses such as motility assays were employed to show consequences of PdeL transcription regulation. My findings established PdeL as a dual-function transcription regulator in *E. coli* (results part 1, publication).

As part of my master thesis I also had addressed the cellular localization of PdeL. By serendipity, cellular localization of PdeL revealed consequences of PdeL DNA-binding on other cellular properties. Therefore, I also analyzed cellular properties and processes such as nucleoid structure and cell division by fluorescence microscopy. My findings established a general understanding of PdeL cellular localization. In addition, the results revealed general considerations that should be

taken into account when DNA-binding proteins such as transcription regulators are analyzed using plasmidic expression systems (results part 2, manuscript).

Lastly, during my work on PdeL I obtained knowledge on bioinformatic analysis and handling of next generation sequencing data (NGS), which I transferred to other projects in the lab. I contributed to an effort to cure *E. coli* strains of FRT-site scars, left in the chromosome in the process of genome editing (results part 3, manuscript).

2. Results

2.1. The Transcription Regulator and c-di-GMP Phosphodiesterase PdeL Represses Motility in *Escherichia coli*

In

Yilmaz, C., Rangarajan, A.A., and Schnetz, K. (2020). The transcription regulator and c-di-GMP phosphodiesterase PdeL represses motility in *Escherichia coli*. J. Bacteriol. 203, JB.00427-00420. https://doi.org/10.1128/JB.00427-20

we characterized PdeL as a novel transcription regulator in *E. coli*.

PdeL is a transcription regulator and cyclic-di-GMP phosphodiesterase in *E. coli*. Positive autoregulation has led to characterization of PdeL as a transcription regulator and suggestion that it is a trigger enzyme in the context of cyclic-di-GMP (Hengge, 2016). In this study we systematically characterized PdeL as a functional transcription regulator. To this end, we tested expression of *pdeL* itself. Analysis of *pdeL* expression will aide in understanding the functional role of PdeL. Next, we addressed the question whether PdeL regulates the expression of additional genes other than its own expression. In addition to identifying and analyzing regulation of target genes by PdeL, we analyzed the role of PdeL transcription regulatory function on flagellar operon *fliFGHIJK* for motility. We approached the characterization of PdeL by employing molecular genetic and genomic analyses.

Expression analyses such as β-galactosidase assays in combination with immunodetection allowed thorough analysis of *pdeL* expression. Our results suggest a 25-fold repression of *pdeL* by the global repressor H-NS. Accordingly, protein levels of PdeL are low but constant throughout different growth phases. Positive autoregulation of PdeL was detectable only when PdeL was provided ectopically at approximately 10-fold higher protein levels than when from the native chromosomal gene. In addition, protein levels of PdeL were independent of elevated cyclic-di-GMP levels. From these results it can be concluded that under the tested conditions PdeL activity is low mainly due to repression of *pdeL* by H-NS and independent of the growth phase and cyclic-

di-GMP level. Furthermore, plasmidic driven synthesis of PdeL, mimicking an activation of *pdeL* expression at around 10-fold, was sufficient for analysis of its transcription regulatory activity.

RNA-sequencing allowed detection of differentially expressed genes in the presence of elevated PdeL levels. Therefore, we identified transcription regulatory targets by short induction of ectopic PdeL expression. Furthermore we characterized regulation of target genes by expression analysis using target gene reporter fusions and *in vitro* DNA-binding assays. To this end, our data show transcription regulation of specific target genes by PdeL including flagellar class 2 operon *fliFGHIJK* and gene *sslE* encoding for a metalloprotease. Specific binding of PdeL to target promoter regions was shown. Further, DNA-binding of the N-terminal $PdeL_{DBD}$ domain was dependent on dimerization of a C-terminal domain. A 4-fold repression of the operon *fliFGHIJK* and a 7.5-fold activation of gene *sslE* suggest both activating and repressing activity by PdeL. In addition, regulation of target genes was shown to be dependent on DNA-binding and independent of enzymatic activity of PdeL. Taken together, it can be concluded that PdeL is a dual-function transcription regulator, capable of activating and repressing specific target genes independent of the enzymatic activity of its PDE-domain. In particular, activation of *sslE* suggests a role of PdeL in virulence of pathogenic *E. coli*. Furthermore, repression of the flagellar class 2 operon *fliFGHIJK*, necessary for synthesis of flagella, by PdeL was expected to affect motility of the bacterium.

Finally, motility assays show that elevated PdeL levels indeed inhibit motility. *In vivo* detection of surface exposed subunits of the flagella suggests an inhibition of flagellar synthesis by PdeL transcription regulatory activity on *fliFGHIJK*. Interestingly, DNA-binding deficient variant $PdeL_{DBD5M}$ was capable of restoring motility when motility was inhibited by elevated cellular cyclic-di-GMP levels. This suggests enzymatic activity of the PDE-domain independent of PdeL DNA-binding. Furthermore, substitution of the native *fliFGHIJK* promoter uncoupled its expression from its physiological regulators such as the flagellar master regulator and activator FlhDC. This uncoupling relieved *fliFGHIJK* from PdeL repression. Together with *in vitro* DNA-binding assay of PdeL at *fliF* promoter region, these findings suggest a direct inhibition of *fliFGHIJK* expression by PdeL or by inhibition of FlhDC dependent activation. Taken together, we conclude that the transcription regulator function of PdeL is epistatic to its function as a

phosphodiesterase. PdeL is capable of DNA-binding necessary for transcriptional regulation of flagellar class 2 operon *fliFGHIJK* as well as hydrolysis of cyclic-di-GMP by its PDE-domain.

In conclusion, we hypothesize that regulation of *fliFGHIJK* operon by PdeL adds one additional level at which *E. coli* can control the transition in its lifestyle from a motile to a sessile one. In particular, PdeL appears to function as a hallmark in controlling the initiation of flagella synthesis as the *fliFGHIJK* operon encodes for flagellar subunits needed in early phase of flagellar synthesis.

Yilmaz et al. (2020) was prepared with contributions by Cihan Yilmaz, Aathmaja Anandhi Rangarajan and Karin Schnetz. Together with K.S. I designed all experiments and performed all experimental tasks except for construction of the donor strain T2517 necessary for fluorescent detection of flagella which was done by A.A.R. K.S. contributed by supervision, in addition to drafting and writing the manuscript. In addition, I contributed to drafting and writing of the manuscript including methodology and data visualization. All authors discussed the model and commented and edited the manuscript.

2.2. High abundance of transcription regulators compacts the nucleoid in *Escherichia coli*

In

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I describe the phenomenon that high abundance of a DNA-binding transcription regulator causes nucleoid condensation, which was observed by serendipity during my work on PdeL cellular localization.

Cellular localization of transcription regulators can be indicative for the physiological function of the protein. Phosphodiesterases have been shown to be acting locally in the context of cyclic-di-GMP signaling (Hengge et al., 2019). To this end, we initially addressed the question where PdeL is localizing within *E. coli*. By serendipity we observed PdeL nucleoid-association to cause nucleoid condensation and thus aimed to thoroughly describe it for a possible PdeL specificity. In order to show PdeL DNA-binding specific nucleoid condensation other transcription regulators were tested. To this end, we approached the analysis of PdeL cellular localization and its effect on nucleoid structure by fluorescence microscopy visualizing proteins MukB and FtsZ, important for structural maintenance of the chromosome and cell division respectively.

During initial studies filamentous growth was observed when PdeL variants capable of DNA-binding were overexpressed. Therefore, we tested localization of fluorescent PdeL-mVenus variants by low induction of a P_{ara} promoter by fluorescence microscopy. Low induction levels were possible by employing a strain with genetically modified arabinose regulon (Breddermann and Schnetz, 2016; Khlebnikov et al., 2000). Detection of nucleoid co-localization was possible by employing a fluorescent variant of the nucleoid associated protein HU subunit HupA. Our data show that PdeL-mVenus is nucleoid-associated and also occupied the whole nucleoid. In addition, a DNA-binding deficient variant, $PdeL_{DBD5M}$-mVenus, did not co-localize with the nucleoid. Interestingly, the area occupied by the nucleoid was different in *E. coli* overexpressing PdeL-mVenus. The nucleoid appeared to occupy a space more centered towards mid-cell while the cell

poles appeared nucleoid free reminiscent of nucleoid condensation. These results suggest that DNA-binding of PdeL changes the area occupied by the nucleoid. This finding further raised the question whether the changes in nucleoid structure were due to changes in the structural maintenance of the chromosome and cell division.

To this end, we tested the localization of hallmark proteins involved in structural maintenance of the chromosome and cell division MukB and FtsZ respectively. Our results show that DNA-binding of elevated levels of PdeL caused the loss of specific MukB-mNG localization. Further, protein levels of MukB were independent of PdeL. In addition, a fluorescent fusion of cell division protein FtsZ-mNG was mispositioned upon PdeL DNA-binding. These results suggest significant changes in the nucleoid structure and subsequent consequences for cell division when PdeL abundance in the cell is increased.

In addition to PdeL DNA-binding, we could also show that additional transcription regulators LacI, RutR, RcsB, LeuO and Cra caused similar changes in nucleoid organization and cell division upon expression at similar levels to PdeL. From these results it can be concluded that it is the DNA-binding of the different transcription regulators at elevated protein levels, which leads to the observed changes in nucleoid organization and cell division by a yet unknown mechanism. Estimation of PdeL abundance in cells with increasingly observable nucleoid condensation suggests possible occupation of a single nucleoid equivalent present in the cell by increasing numbers of PdeL dimers respectively.

Our data further shows that inhibition of specific DNA-binding of LacI upon association of IPTG did not abolish its effect on nucleoid organization. Thus it can be concluded that, for LacI, non-specific DNA-binding appears to be the reason for its effect on nucleoid organization and cell division.

Taken together, our data suggests that at elevated protein levels, DNA-binding of the tested transcription regulators affects nucleoid organization and cell division. We hypothesize that the nucleoid is also increasingly occupied with increased levels of DNA-binding protein. In addition, we conclude that the nucleoid occlusion system is responsible for the shift in positioning of the Z-ring. When the nucleoid is condensed to mid cell, the nucleoid occlusion system ensures that

no Z-ring can be formed over the area occupied by the nucleoid. Therefore the Z-ring is shifted closer to the cell poles not occupied by the nucleoid. Further, these findings have implications for analysis on transcription regulators in general. Approaches such as ectopically provided expression might already lead to observations in the context of nucleoid structure and cell division which are essentially caused by occupation of the nucleoid by the expressed DNA-binding protein.

This manuscript was prepared with contributions of Cihan Yilmaz and Karin Schnetz. The study was conceived and designed by both authors. I performed all experiments and the results were discussed by both authors. I wrote the draft of the manuscript and made all figures. Karin Schnetz commented and edited the manuscript.

2.3. Deletion of FRT-sites by no-Scar recombineering in *Escherichia coli*

In

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we adapted published methods for Cas9-mediated recombineering for deletion of FRT-sites in *E. coli*.

During the process of lambda Red mediated recombination, used for genome engineering, resistance cassettes are used for positive selection of constructs (Datsenko and Wanner, 2000). These resistance cassettes are flanked by FRT-sites for later targeting by the yeast FLP recombinase. As a result of yeast FLP recombinase activity, one FRT-scar remains in the genome. The insertion of FRT-scars into the genome can have different consequences. First, the genomic context of a locus can be changed and have effects on the regulation of a loci. Second, remnant FRT-scars which are in close proximity to each other can be recognized as targets by the yeast FLP recombinase and cause larger deletions of genomic DNA regions. We addressed the question whether published methods of Cas9-mediated recombineering can be utilized to cure FRT-scars in an *E. coli* strain, already genetically modified at one or two sites. The deletion of FRT-sites was hence approached by targeting of the Cas9 endonuclease to loci specific FRT-sites by employing $sgRNA_{FRT}$.

Our results show that loci specific FRT-sites can be deleted by the no-Scar recombineering system. In addition, targeting of a single FRT-site was more efficient than simultaneous targeting of two FRT-sites within one recombination step. Further, genomic sequencing has shown genomic integrity after no-Scar recombineering.

This manuscript is of importance as curing of FRT-sites aids in restoring the native context of loci. Further it allows multiple steps of genome engineering approaches which allow the construction of specified *E. coli* strains.

The manuscript was prepared with contributions by Aathmaja Anandhi Rangarajan, Cihan Yilmaz and Karin Schnetz. A.A.R. and K.S. conceived and designed the study to adapt published protocols

for Scarless Cas9 assisted recombineering. I contributed by bioinformatics analysis of genomic sequencing data using breseq software (Deatherage and Barrick, 2014). K.S. drafted the manuscript and all authors commented and edited the manuscript.

3. Discussion

In this thesis I established a detailed understanding of the transcription regulator and cyclic-di-GMP phosphodiesterase PdeL. Starting from its initial use as a purifiable phosphodiesterase (Schmidt et al., 2005) to its proposal as one of many trigger enzymes (Hengge, 2016), this thesis has added novel insight into this interesting transcription regulator and cyclic-di-GMP phosphodiesterase in *E. coli*. In the following sections I will discuss the results of my thesis and their implications for our understanding of PdeL, its physiological importance in different processes and provide an outlook on how to approach open questions.

3.1. Cyclic-di-GMP responsive transcription regulators and PdeL

Cyclic-di-GMP has been shown to function as an effector molecule influencing the DNA-binding of transcription regulators, defined as cyclic-di-GMP responsive transcription regulators. For example, binding of cyclic-di-GMP to *Streptomyces* transcription regulator BldD induces formation of a dimer enabling BldD binding to target promoter sequence (Schumacher et al., 2017). Binding of cyclic-di-GMP to BldD occurs at specific sites that are characteristic for cyclic-di-GMP binding proteins (Amikam and Galperin, 2006; Chou and Galperin, 2016; Navarro et al., 2009). As a phosphodiesterase, PdeL also binds cyclic-di-GMP. This raises the question whether cyclic-di-GMP is important for controlling the activity of PdeL as a transcription regulator allowing classification of PdeL as a cyclic-di-GMP responsive transcription regulator.

The unique domain structure of PdeL in comparison to the 12 other phosphodiesterases in *E. coli* is the presence of an N-terminal DNA-binding domain (DBD) of the FixJ/NarL-type. DNA-binding by PdeL requires a dimerization domain, which may have a regulatory role similar to receiver domains of two-component response regulators (Maris et al., 2002; Yilmaz et al., 2020). I could show that the presence of $PdeL_{EAL}$ domain or another dimerization domain such as the phage Lambda repressor cI dimerization domain is needed and sufficient for DNA-binding of PdeL (Yilmaz et al., 2020). Thus, $PdeL_{EAL}$ serves its enzymatic phosphodiesterase purpose as well as a dimerization domain for DNA-binding of PdeL. It is an open question whether the PDE-domain functions as a regulatory domain. Such a function would fit well with the finding that the PDE-domain is in a monomer to dimer equilibrium which is shifted towards the dimeric state upon

binding of cyclic-di-GMP to $PdeL_{EAL}$ (Sundriyal et al., 2014). However, an enzymatically inactive mutant of PdeL able to bind cyclic-di-GMP was described previously (Sundriyal et al., 2014). We could show that this enzymatically inactive $PdeL_{D263N}$ mutant was still capable of inhibiting motility (Yilmaz et al., 2020). Further, *in vitro* DNA-binding of PdeL and $PdeL_{EVL\text{-}AAA}$ to the *fliF* promoter fragment was independent of cyclic-di-GMP (data not shown). Mutagenesis of the EVL-motif to three alanines presumptively does not prevent association of cyclic-di-GMP but render the PDE enzymatically inactive. Further, the transcription regulatory function of PdeL, including autoregulation, was independent of its ability to hydrolyze cyclic-di-GMP as shown for PdeL and $PdeL_{EVL\text{-}AAA}$ (Yilmaz et al., 2020). We also showed that protein levels of PdeL were independent of cellular cyclic-di-GMP levels (Yilmaz et al., 2020). Thus, a cyclic-di-GMP responsiveness by altering the protein level is unlikely.

One approach to identify possible cyclic-di-GMP responsiveness of PdeL would be to perform transcriptomic analysis under cyclic-di-GMP elevated conditions. Elevated cyclic-di-GMP levels can be achieved by mutation of the *pdeH* gene encoding the major phosphodiesterase PdeH (Reinders et al., 2016). Under these conditions binding of cylic-di-GMP can occur under physiological conditions circumventing possible limitations present in our initial *in vitro* DNA-binding assay. Reporters encompassing promoters of genes *fliF* and *sslE* which we have shown to be regulated by PdeL can be utilized for expression analysis. A significantly different regulation of P_{fliF} and P_{sslE} could indicate a cyclic-di-GMP responsiveness of PdeL on the level of its transcription regulatory activity.

3.2. Integrating PdeL into the hierarchical regulation of motility in *E. coli*

The hierarchical regulation of motility and flagellar synthesis in general is very interesting and complex (Fig. 1A). The class 1 transcription activator and master flagellar regulator FlhDC controls all class 2 operons which are transcribed by RNA Polymerase sigma 70 holoenzyme. Release of the alternative sigma factor FliA from its anti-sigma factor FlgM is controlled by the progress of flagellar synthesis (Fitzgerald et al., 2014). The anti-sigma factor FlgM is exported out of the cell thus relieving FliA inhibition upon assembly of the export apparatus, as part of FlhDC activated class 2 genes, (Fitzgerald et al., 2014; Kutsukake, 1994). Further, class 2/3 genes are regulated

by FlhDC and transcribed by RNA polymerase sigma 70 or sigma 28 holoenzyme (Fig. 1A). Transcription of class 3 genes by the RNA polymerase Sigma 28 holo-enzyme mediates a transition from early to late flagellar synthesis phase (Fitzgerald et al., 2014). The diversification into different classes of operons and genes thus can follow a hierarchical order. By transcriptome analysis we could show that PdeL represses expression of the *fliFGHIJK* operon (Yilmaz et al., 2020). How can the regulation of flagellar class 2 operon *fliFGHIJK* by PdeL be integrated into this well-ordered system of flagellar synthesis?

Regulation of *flhDC* is performed by multiple transcription regulators such as H-NS, LrhA, OmpR and CAP (Lee and Park, 2013). The *fliFGHIJK* operon is one of multiple class 2 flagellar operons regulated by the master regulator FlhDC (Fitzgerald et al., 2014). Our findings show that PdeL, by direct repression or inhibition of FlhDC dependent activation, represses *fliFGHIJK* expression (Yilmaz et al., 2020).

We hypothesize that by regulation of an early expressed class 2 flagellar operon, PdeL can fine tune the decision of the bacteria to initiate synthesis of new flagella. The synthesis of subunits, encoded by the *fliFGHIJK* operon, required for the assembly of the MS-ring in the inner membrane can function well as a point of regulation. In theory once synthesis of a flagellum is initiated, master regulator FlhDC activates expression of early flagellar synthesis genes and thus the assembly of the basal body and export apparatus (Fitzgerald et al., 2014). The export apparatus reliefs the alternative sigma factor of inhibition by anti-sigma factor FlgM by exporting it out of the cell (Kutsukake, 1994). Next, FlhDC and the new free alternative sigma factor FliA can activate expression of late flagellar assembly genes (Fig. 1). Repression of *fliFGHIJK* by PdeL at this stage of the flagellar assembly would allow ongoing syntheses to finish without the initiation of new basal body assemblies.

The exact mechanism by which bacteria control the number of total flagella on the surface of bacteria is not yet fully understood (Schuhmacher et al., 2015). Staining of FlgE, a late assembled flagellar subunit, showed that the number of fully assembled flagella was reduced, but not fully abolished, when PdeL was overexpressed (Yilmaz et al., 2020). Regulation of other flagellar synthesis genes by PdeL should lead to no detectable flagella. Control on the level of initiation of flagellar synthesis might be a suitable candidate for control of flagella number. Control of flagellar

synthesis initiation is supported by *E. coli´*s effort to efficiently use resources. We could show that binding of PdeL to *fliF* promoter region overlaps with binding sites of FlhDC and CRP. Thus, repression of *fliFGHIJK* by PdeL can either be direct or indirect by interfering with its activation by FlhDC. Taken together, PdeL´s role in inhibiting motility by repressing the expression of flagellar subunits *fliFGHIJK* might be a mechanism *E. coli* employs to control the number of flagella that is synthesized.

Interestingly, the importance of repression of *fliFGHIJK* by PdeL contrasts with its function as a cyclic-di-GMP phosphodiesterase. Our data shows, that under the conditions tested, the DNA-binding function of PdeL dominates its phosphodiesterase activity. In addition, enzymatic activity of DNA-binding deficient $PdeL_{DBD5M}$ mutant was sufficient to restore motility inhibited by cyclic-di-GMP (Yilmaz et al., 2020). $PdeL_{DBD5M}$ does so by hydrolyzing cyclic-di-GMP, which otherwise associates with motor brake protein YcgR and inhibits motility (Boehm et al., 2010; Fang and Gomelsky, 2010; Paul et al., 2010).

Further efforts on whether PdeL inhibits transcription initiation at the *fliF* promoter region by interfering with FlhDC activity or RNA-Polymerase binding will help understanding its role in motility inhibition. Additional *in vitro* studies could identify possible interaction of PdeL with FlhDC at the *fliF* promoter region and elucidate whether PdeL represses *fliF* by inhibiting its activation by FlhDC.

3.3. Is PdeL important for virulence in pathogenic *E. coli* strains?

Although all work was conducted with the non-pathogenic strain *E. coli* K-12, activation of *sslE* expression by PdeL adds an interesting aspect. E. coli K-12 lacks the type 2 secretion system important for its pathogenicity (Patrick et al., 2010). SslE has been extensively studied in enterotoxigenic *Escherichia coli* strain H10407 (Luo et al., 2014). In mouse models, immunization with SslE led to protection from infections by extraintestinal pathogenic strains of *E. coli* (Nesta et al., 2014). In wake of these interesting findings, the question arises whether PdeL could indeed play a role in virulence of pathogenic *E. coli* strains.

I also showed that PdeL can activate the expression of metalloprotease and virulence factor *sslE* (Yilmaz et al., 2020). Sequence comparison between *E. coli* K-12 and ETEC strain H10407 showed

that in both strains very high (>99%) sequence identity exists between *pdeL* as well as *sslE* and its regulatory region (Johnson et al., 2008; UniProt, 2021). Luo et al. (2014) postulate *sslE* as contributing to pathogenesis. Specifically, *sslE* was shown to be degrading intestinal mucin and thus mediating the delivery of toxins during infection (Luo et al., 2014). Taken together, activation of *sslE* by PdeL, potentially adds virulence to the pathways PdeL might be involved in. Regulation of *sslE* expression is yet poorly understood. I was able to narrow down the DNA-binding site of PdeL at *sslE* promoter region (Yilmaz et al., 2020). Analysis of the transcriptional regulation of *sslE* in *E. coli* K-12 might prove useful in better understanding its role in pathogenic *E. coli* strains. One approach to gain further insight into the expression regulation of *sslE* would be the mapping of transcription start site by 5´-RACE. Mapping of the transcription start site would narrow down the promoter region which could then be used to perform a promoter pull-down assay to identify other possible transcription regulators bound (Chaparian and van Kessel, 2021). The reporter fusion of *sslE* used to analyze its regulation by PdeL can also be further utilized (Yilmaz et al., 2020). A P_{sslE} *lacZ* or P_{sslE} *mNeonGreen* reporter can thus be employed to screen for different conditions such as pH or temperature which alter the activity of P_{sslE}. In addition, a genome library screen employing the P_{sslE} *lacZ* reporter could be useful in identifying additional transcription regulators of *sslE*.

3.4. When is *pdeL* expressed?

Central to understanding the physiological function of PdeL is to understand when *pdeL* is expressed. Expression analysis of *pdeL* has shown strong repression by the global repressor H-NS (Yilmaz et al., 2020). A question that should thus be addressed is the physiological condition under which *pdeL* expression or maybe PdeL itself is activated.

We have shown that activity of P_{pdeL} is independent of the growth phase, and that the level of natively expressed PdeL-3xFLAG were constant at laboratory growth conditions (Yilmaz et al., 2020). In addition, we have shown that PdeL-3xFLAG is very stable (Yilmaz and Schnetz, unpublished). Protein levels of PdeL-3xFLAG and its enzymatically inactive variant $PdeL_{EVL\text{-}AAA}$-3xFLAG were approximately similar 160 minutes after inhibition of translation. In theory, high protein stability should suffice for produced PdeL to be sustained throughout multiple

generations in case of no specific degradation. Cellular PdeL levels can be ectopically increased by utilization of a plasmidic P_{tac} *pdeL* fusion. Plasmid driven synthesis of PdeL by basal activity of uninduced P_{tac} was shown to approximately 10-fold higher than natively synthesized PdeL (Yilmaz et al., 2020). In addition, elevation of PdeL levels by 10-fold using basal activity of P_{tac} *pdeL* was sufficient for repression of *fliF* by PdeL (Yilmaz et al., 2020). These results suggest that a small but significant upregulation of PdeL expression might already suffice for a regulatory effect. In addition, it might also be possible that low amounts of PdeL that are readily detectable in PdeL are already sufficient but lack an activation as discussed in the context of putative cyclic-di-GMP responsiveness of PdeL. Although PdeL is an atypical member of the FixJ/NarL family of DNA-binding proteins, an approach to identify possible post-translational modification of PdeL such as phosphorylation could reveal new insight.

Further, I could detect the positive autoregulation of PdeL to be around four-fold only when PdeL was overexpressed ectopically, in contrast to published data (Reinders et al., 2016). SELEX-DNA-binding studies have suggested the presence of Cra and Nac binding sites in the *pdeL* promoter region (Aquino et al., 2017; Shimada et al., 2005). Expression of *pdeL* was repressed around two-fold by Nac (Aquino et al., 2017). Additional studies on *pdeL* repression by Nac might reveal a possible crosstalk between nitrogen metabolism and *pdeL* expression. Promoter region binding of the transcription regulator Cra is inhibited by association of fructose 1-phosphate (Bledig et al., 1996). It remains unclear whether Cra can function as an activator or repressor for *pdeL* activity. Initial studies showed P_{pdeL} activity to be independent of the presence of fructose which would inhibit putative Cra binding and thus regulatory activity (data not shown).

One important factor in the low abundancy of PdeL in *E. coli* is the approximately 25-fold repression of *pdeL* by H-NS (Yilmaz et al., 2020). Possible transcriptional regulators of *pdeL* would have to overcome *pdeL* repression by H-NS. Different mechanisms have been postulated able to overcome repression of genes by H-NS. Activity of P_{pdeL} might be upregulated by a transcription regulator that functions as an H-NS antagonist. One possible candidate might be a transcriptional activator, which competes for binding to *pdeL* promoter region and thus reliefs it of H-NS repression. Beyond the proposed positive autoregulation of PdeL by Reinders et. al (2016) no transcriptional activator of P_{pdeL} has been characterized yet. Degradation of H-NS by the Lon

protease under acidic conditions in presence of low amounts of magnesium has been shown to activate the expression of H-NS repressed genes in *S. enterica* serovar Typhimurium (Choi and Groisman, 2020). The degradation of H-NS might also serve in regulating *pdeL* expression in contrast to activation by an H-NS antagonizing transcription regulator. A transposon mutagenesis screening could be employed to create conditions where activity of P_{pdeL} is increased. Possible outcomes could be the increased expression of a P_{pdeL} activating transcription regulator. In addition to a transposon screening, a P_{pdeL} *lacZ* reporter could be used for testing different conditions. These conditions could encompass temperature and osmolality in addition to the discussed low pH with additional magnesium present for Lon protease dependent degradation of H-NS (Choi and Groisman, 2020).

In addition, expression of phosphodiesterase PdeH has been shown to be dependent on FliA (σ^{28}) (Fig. 1B). One interesting approach would be to check the presence of FliA (σ^{28}) dependent transcription initiation of *pdeL* in addition to σ^{70}. Transition of flagellar synthesis to later stages, initiated by the relief of FliA (σ^{28}) inhibition by FlgM could function as a hallmark to finish ongoing assembly of flagella without initiating new ones by utilizing PdeL mediated repression of *fliFGHIJK*.

3.5. DNA-binding of transcription regulators causes nucleoid compaction

In this thesis plasmidic expression systems were employed to provide PdeL for its functional analyses. In detail, I used plasmids that carry *pdeL* either under the control of P_{tac} or P_{ara} and induced activity by addition of the respective inducers. One factor driving the synthesis rate of *pdeL* under the respective promoters is the strength of the promoter and the copy number of the plasmid. During initial efforts to characterize PdeL, a growth inhibition was observed when plasmidic expression directed by P_{tac} was fully induced using a p15A-derived low to medium-copy number plasmid (data not shown). To circumvent the growth inhibition upon PdeL overexpression two strategies were adopted. First, PdeL was provided by the basal activity of the P_{tac} promoter. In this case, approximately 10-fold more PdeL was synthesized as compared to the native *pdeL* gene (Yilmaz et al., 2020). Repression of *fliF* was detected by these basal P_{tac}-directed PdeL levels. The second strategy employed was the use of the P_{ara} promoter. The activity

of the P_{ara} promoter is finely tunable by addition of arabinose, which triggers activation of P_{ara} by AraC. The expression of *pdeL* under the control of the P_{ara} promoter was used in (Yilmaz et al., 2020) and even more extensively in the second manuscript on describing the adverse effects of DNA-binding on nucleoid structure and cell division.

Fluorescent fusion of PdeL, PdeL-mVenus co-localized with the nucleoid. More specifically, co-localization of PdeL-mVenus to the nucleoid, as visualized by HupA-mCherry, covered the whole nucleoid. This is indicative for PdeL not only binding to specific sites at *fliF* and *sslE* promoter regions as discussed in (Yilmaz et al., 2020) but also to unspecific sites. Upon expression of PdeL-mVenus the nucleoid was spatially limited to an area at midcell.

We tested the localization of structural maintenance of chromosome proteins, as condensation of nucleoid to midcell is indicative of defects in nucleoid organization and possibly segregation. To avoid artificial observations and verify its specificity for PdeL expression, I also tested additional transcription regulators. The scope of tested transcription regulators included transcription regulators RcsB, LacI, RutR, LeuO, and Cra. These transcription regulators were chosen based on their individual DNA-binding affinities and abundance (Shimada et al., 2018). Localization of structural maintenance of chromosome protein MukB changed upon overexpression of PdeL and the other transcription regulators as visualized by fluorescence microscopy. Interestingly, I could show that loss of MukB co-localization with the nucleoid was not due to changes in MukB protein level in case of PdeL expression. This result is indicative of possible defects in nucleoid segregation causing the nucleoid to remain in a condensed conformation at midcell. Defects in nucleoid segregation can be tested by detection of the *ter* region of sister chromatids *in vivo*. Insertion of multiple *lacO* operator site repeats has been used to localize chromosome regions (Lau et al., 2003). Binding of fluorescent tagged LacI to multiple *lacO* operator sites within the *ter* region can thus be used to detect segregation of sister chromatids by fluorescence microscopy. Therefore, defects in nucleoid segregation would cause *ter* regions to remain in proximity without segregating.

Negative regulatory systems enable coordination of the cell division site formed by the Z-ring. The nucleoid occlusion system, mediated by SlmA, is known to exclude Z-ring formation over nucleoid containing areas (Cho et al., 2011). We tested whether the condensation of the nucleoid

to midcell also caused the exclusion of Z-ring formation from the nucleoid occupied midcell. Indeed, condensation of the nucleoid to midcell led to positioning of the Z-ring closer towards the cell poles upon expression of PdeL and other transcription regulators. This is indicative for the bacteria responding to nucleoid condensation introduced by expression of PdeL by rearranging, possibly even stalling, formation of the Z-ring required for cell division. The role of the nucleoid occlusion system in protecting the bacteria from even more adverse effects of PdeL DNA-binding can be tested in a genetic background lacking *slmA*. I hypothesize that lack of the nucleoid occlusion system will cause PdeL expression to lead to formation of non-viable cells after cell division. Formation of non-viable daughter cells can arise from cell division damaging the nucleoid as the Z-ring will be able to form over the nucleoid.

In particular, LacI was tested for its specific and non-specific DNA-binding. Association of IPTG to LacI inhibits specific DNA-binding by introduction of structural changes. LacI-IPTG still caused changes in nucleoid structure. Additional work on differentiating specific and non-specific DNA-binding of other transcription regulators will support the hypothesis of non-specific DNA-binding induced adverse effects on nucleoid structure. For some of the tested transcription regulators effector molecules and conditions inhibiting specific DNA-binding have been identified (Shimada et al., 2007).

We have shown that in presence of high levels, DNA-binding proteins can cause changes in the nucleoid structure. This could be narrowed down to non-specific DNA-binding in case of LacI. The exact mechanism leading to the observed changes are subject to further analysis. I hypothesize that physically covering the nucleoid with DNA-binding proteins interferes with molecular processes requiring access to the nucleoid. I hypothesize that physically covering the nucleoid with DNA-binding proteins interferes with the molecular processes which require access to the nucleoid. If processes such as transcription and replication are inhibited by occupation of the nucleoid, stalling of downstream processes such as cell division are necessary to ensure proliferation. One such example could be the regulatory system coordinating replication and cell division. In case of stalled segregation of the sister chromosomes, which leaves the nucleoid condensed at midcell, formation of the Z-ring is shifted away from the nucleoid occupied midcell area.

Taken together, during studies on DNA-binding proteins, additional steps of precautions must be taken in order to avoid the occurrence of phenotypes which are essentially artifacts. Depending on the approach employed, the synthesis rate of the DNA-binding protein and its stability must be considered when expressed ectopically. The synthesis rate is in part determined by the copy number of the plasmid, the promoter, the induction level and the duration of expression. We have shown that microscopy can be employed as a simple tool to test for unwanted artifacts. These artifacts may also have implications on genome wide transcriptome analyses. Supporting data are thus necessary when genome wide binding analysis ChIP-seq or RNA-seq suggests interaction of a transcription regulator or regulation of multiple loci. Identification of physiological conditions under which the expression of the gene and protein of interest is activated might ease this problem, although not trivial. The use of chromosomal integrations with an additional copy of the gene of interest might be useful.

In particular, the lack of knowledge on regulation of *pdeL* led to our serendipitous observation. For thorough characterization of PdeL further insight into the regulation of *pdeL* expression has become of utter importance. Identification of the mechanism by which activation of PdeL occurs, by yet unknown transcriptional or post-transcriptional means, will aid integrating PdeL into a physiological niche. There is room for speculation in the intriguing context PdeL is integrated in as a transcription regulator and cyclic-di-GMP phosphodiesterase. We have shown that DNA-binding of PdeL is dependent on dimerization at its C-terminus. Binding of cyclic-di-GMP to $PdeL_{EAL}$ as its substrate for hydrolysis is only going to occur in a timely limited manner. If the PdeL transcription regulatory function was to respond to cyclic-di-GMP at its PDE-domain, the kinetics of its enzymatic activity could be determining the duration of the cyclic-di-GMP binding induced signal. No direct comparison of enzymatic activity of PdeL to other phosphodiesterases present in *E. coli* has been performed yet. Interestingly, PdeL harbors an alanine to valine exchange within the characteristic EAL-motif of its PDE-domain. In theory, a strategy of low enzymatic activity could be employed by PdeL for prolonging the duration of cyclic-di-GMP association before its hydrolysis to two GMP molecules.

A cyclic-di-GMP responsiveness of PdeL would add to the complexity of cyclic-di-GMP signaling. Our current understanding is that transcription regulatory function of PdeL dominates its

phosphodiesterase activity. This results in a model where PdeL inhibits motility by transcription regulatory activity and is also capable of hydrolyzing cyclic-di-GMP which is otherwise inhibiting motility.

4. References

Aki, T., Choy, H.E., and Adhya, S. (1996). Histone-like protein HU as a specific transcriptional regulator: co-factor role in repression of gal transcription by GAL repressor. Genes Cells *1*, 179-188.

Altegoer, F., Schuhmacher, J., Pausch, P., and Bange, G. (2014). From molecular evolution to biobricks and synthetic modules: a lesson by the bacterial flagellum. Biotechnol. Genet. Eng. Rev. *30*, 49-64.

Amikam, D., and Galperin, M.Y. (2006). PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics *22*, 3-6.

Aquino, P., Honda, B., Jaini, S., Lyubetskaya, A., Hosur, K., Chiu, J.G., Ekladious, I., Hu, D., Jin, L., Sayeg, M.K*., et al.* (2017). Coordinated regulation of acid resistance in Escherichia coli. BMC. Syst. Biol. *11*, 1.

Bernhardt, T.G., and de Boer, P.A. (2005). SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over Chromosomes in E. coli. Mol. Cell. *18*, 555-564.

Bledig, S.A., Ramseier, T.M., and Saier, M.H., Jr. (1996). Frur mediates catabolite activation of pyruvate kinase (pykF) gene expression in Escherichia coli. J. Bacteriol. *178*, 280-283.

Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C.A., Ackermann, M., Kaever, V., Sourjik, V., Roth, V., and Jenal, U. (2010). Second Messenger-Mediated Adjustment of Bacterial Swimming Velocity. Cell *141*, 107-116.

Breddermann, H., and Schnetz, K. (2016). Correlation of Antagonistic Regulation of leuO Transcription with the Cellular Levels of BglJ-RcsB and LeuO in Escherichia coli. Front. Cel.l Infect. Microbiol. *6*, 106.

Browning, D.F., and Busby, S.J. (2016). Local and global regulation of transcription initiation in bacteria. Nat. Rev. Microbiol. *14*, 638-650.

Busby, S.J.W. (2019). Transcription activation in bacteria: ancient and modern. Microbiology *165*, 386-395.

Chaparian, R.R., and van Kessel, J.C. (2021). Promoter Pull-Down Assay: A Biochemical Screen for DNA-Binding Proteins. Methods Mol. Biol. *2346*, 165-172.

Cho, H., McManus, H.R., Dove, S.L., and Bernhardt, T.G. (2011). Nucleoid occlusion factor SlmA is a DNA-activated FtsZ polymerization antagonist. Proc. Natl. Acad. Sci. U.S.A. *108*, 3773-3778.

Choi, J., and Groisman, E.A. (2020). Salmonella expresses foreign genes during infection by degrading their silencer. Proc. Natl. Acad. Sci. U.S.A. *117*, 8074-8082.

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

Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M., and Finlay, B.B. (2013). Recent advances in understanding enteric pathogenic Escherichia coli. Clin. Microbiol. Rev. *26*, 822-880.

D'Argenio, D.A., and Miller, S.I. (2004). Cyclic di-GMP as a bacterial second messenger. Microbiology *150*, 2497-2502.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. *97*, 6640-6645.

Deatherage, D.E., and Barrick, J.E. (2014). Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. Methods Mol. Biol. *1151*, 165-188.

Dillon, S.C., and Dorman, C.J. (2010). Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. Nat. Rev. Microbiol. *8*, 185-195.

Dorman, C.J., Schumacher, M.A., Bush, M.J., Brennan, R.G., and Buttner, M.J. (2020). When is a transcription factor a NAP? Curr. Opin. Microbiol. *55*, 26-33.

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

Fitzgerald, D.M., Bonocora, R.P., and Wade, J.T. (2014). Comprehensive mapping of the Escherichia coli flagellar regulatory network. PLoS Genet. *10*, e1004649.

Galperin, M.Y. (2010). Diversity of structure and function of response regulator output domains. Curr. Opin. Microbiol. *13*, 150-159.

Gottesman, S. (2019). Trouble is coming: Signaling pathways that regulate general stress responses in bacteria. J Biol. Chem. *294*, 11685-11700.

Grainger, D.C. (2016). Structure and function of bacterial H-NS protein. Biochem. Soc. Trans. *44*, 1561-1569.

Hajduk, I.V., Rodrigues, C.D., and Harry, E.J. (2016). Connecting the dots of the bacterial cell cycle: Coordinating chromosome replication and segregation with cell division. Semin. Cell Dev. Biol. *53*, 2-9.

Hammel, M., Amlanjyoti, D., Reyes, F.E., Chen, J.H., Parpana, R., Tang, H.Y., Larabell, C.A., Tainer, J.A., and Adhya, S. (2016). HU multimerization shift controls nucleoid compaction. Sci. Adv. *2*, e1600650.

Helmann, J.D. (2019). Where to begin? Sigma factors and the selectivity of transcription initiation in bacteria. Mol. Microbiol. *112*, 335-347.

Hengge, R. (2016). Trigger phosphodiesterases as a novel class of c-di-GMP effector proteins. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *371*.

Hengge, R. (2020). Linking bacterial growth, survival, and multicellularity - small signaling molecules as triggers and drivers. Curr. Opin. Microbiol. *55*, 57-66.

Hengge, R., Galperin, M.Y., Ghigo, J.M., Gomelsky, M., Green, J., Hughes, K.T., Jenal, U., and Landini, P. (2016). Systematic Nomenclature for GGDEF and EAL Domain-Containing Cyclic Di-GMP Turnover Proteins of Escherichia coli. J. Bacteriol. *198*, 7-11.

Hengge, R., Haussler, S., Pruteanu, M., Stulke, J., Tschowri, N., and Turgay, K. (2019). Recent Advances and Current Trends in Nucleotide Second Messenger Signaling in Bacteria. J. Mol. Biol. *431*, 908-927.

Hiraga, S., Niki, H., Imamura, R., Ogura, T., Yamanaka, K., Feng, J., Ezaki, B., and Jaffe, A. (1991). Mutants defective in chromosome partitioning in E. coli. Res. Microbiol. *142*, 189-194.

Hiraga, S., Niki, H., Ogura, T., Ichinose, C., Mori, H., Ezaki, B., and Jaffe, A. (1989). Chromosome partitioning in Escherichia coli: novel mutants producing anucleate cells. J. Bacteriol. *171*, 1496-1505.

Hommais, F., Krin, E., Laurent-Winter, C., Soutourina, O., Malpertuy, A., Le Caer, J.P., Danchin, A., and Bertin, P. (2001). Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. Mol. Microbiol. *40*, 20-36.

Ishihama, A., Shimada, T., and Yamazaki, Y. (2016). Transcription profile of Escherichia coli: genomic SELEX search for regulatory targets of transcription factors. Nucleic Acids Res. *44*, 2058-2074.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., and Madden, T.L. (2008). NCBI BLAST: a better web interface. Nucleic Acids Res. *36*, W5-9.

Kearns, D.B. (2010). A field guide to bacterial swarming motility. Nat. Rev. Microbiol. *8*, 634-644.

Khlebnikov, A., Risa, O., Skaug, T., Carrier, T.A., and Keasling, J.D. (2000). Regulatable arabinose-inducible gene expression system with consistent control in all cells of a culture. J. Bacteriol. *182*, 7029-7034.

Kleckner, N.E., Chatzi, K., White, M.A., Fisher, J.K., and Stouf, M. (2018). Coordination of Growth, Chromosome Replication/Segregation, and Cell Division in E. coli. Front. Microbiol. *9*, 1469.

Kutsukake, K. (1994). Excretion of the anti-sigma factor through a flagellar substructure couples flagellar gene expression with flagellar assembly in Salmonella typhimurium. Mol. Gen. Genet. *243*, 605-612.

Lau, I.F., Filipe, S.R., Soballe, B., Okstad, O.A., Barre, F.X., and Sherratt, D.J. (2003). Spatial and temporal organization of replicating Escherichia coli chromosomes. Mol. Microbiol. *49*, 731-743.

Lee, C., and Park, C. (2013). Mutations upregulating the flhDC operon of Escherichia coli K-12. J Microbiol. *51*, 140-144.

Lee, D.J., Minchin, S.D., and Busby, S.J. (2012). Activating transcription in bacteria. Annu. Rev. Microbol. *66*, 125-152.

Lewis, M. (2005). The lac repressor. C. R. Biol. *328*, 521-548.

Lewis, M. (2011). A tale of two repressors. J. Mol. Biol. *409*, 14-27.

Lioy, V.S., Cournac, A., Marbouty, M., Duigou, S., Mozziconacci, J., Espeli, O., Boccard, F., and Koszul, R. (2018). Multiscale Structuring of the E. coli Chromosome by Nucleoid-Associated and Condensin Proteins. Cell *172*, 771-783 e718.

Lucchini, S., Rowley, G., Goldberg, M.D., Hurd, D., Harrison, M., and Hinton, J.C. (2006). H-NS mediates the silencing of laterally acquired genes in bacteria. PLoS Pathog. *2*, e81.

Luo, Q., Kumar, P., Vickers, T.J., Sheikh, A., Lewis, W.G., Rasko, D.A., Sistrunk, J., and Fleckenstein, J.M. (2014). Enterotoxigenic Escherichia coli secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. Infect. Immun. *82*, 509-521.

Majors, J. (1975). Specific binding of CAP factor to lac promoter DNA. Nature *256*, 672-674.

Makela, J., and Sherratt, D.J. (2020). Organization of the Escherichia coli Chromosome by a MukBEF Axial Core. Mol. Cell. *78*, 250-260 e255.

Maris, A.E., Sawaya, M.R., Kaczor-Grzeskowiak, M., Jarvis, M.R., Bearson, S.M., Kopka, M.L., Schroder, I., Gunsalus, R.P., and Dickerson, R.E. (2002). Dimerization allows DNA target site recognition by the NarL response regulator. Nat. Struct. Biol. *9*, 771-778.

Mejia-Almonte, C., Busby, S.J.W., Wade, J.T., van Helden, J., Arkin, A.P., Stormo, G.D., Eilbeck, K., Palsson, B.O., Galagan, J.E., and Collado-Vides, J. (2020). Redefining fundamental concepts of transcription initiation in bacteria. Nat. Rev. Genet. *21*, 699-714.

Navarre, W.W. (2016). The Impact of Gene Silencing on Horizontal Gene Transfer and Bacterial Evolution. Adv. Microb. Physiol. *69*, 157-186.

Navarre, W.W., Porwollik, S., Wang, Y., McClelland, M., Rosen, H., Libby, S.J., and Fang, F.C. (2006). Selective silencing of foreign DNA with low GC content by the H-NS protein in Salmonella. Science *313*, 236-238.

Navarro, M.V., De, N., Bae, N., Wang, Q., and Sondermann, H. (2009). Structural analysis of the GGDEF-EAL domain-containing c-di-GMP receptor FimX. Structure *17*, 1104-1116.

Nesta, B., Valeri, M., Spagnuolo, A., Rosini, R., Mora, M., Donato, P., Alteri, C.J., Del Vecchio, M., Buccato, S., Pezzicoli, A.*, et al.* (2014). SslE elicits functional antibodies that impair in vitro mucinase activity and in vivo colonization by both intestinal and extraintestinal Escherichia coli strains. PLoS Pathog. *10*, e1004124.

Nolivos, S., Upton, A.L., Badrinarayanan, A., Muller, J., Zawadzka, K., Wiktor, J., Gill, A., Arciszewska, L., Nicolas, E., and Sherratt, D. (2016). MatP regulates the coordinated action of topoisomerase IV and MukBEF in chromosome segregation. Nat. Commun. *7*, 10466.

Patrick, M., Gray, M.D., Sandkvist, M., and Johnson, T.L. (2010). Type II Secretion in Escherichia coli. EcoSal Plus *4*.

Paul, K., Nieto, V., Carlquist, W.C., Blair, D.F., and Harshey, R.M. (2010). The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a "backstop brake" mechanism. Mol. Cell. *38*, 128-139.

Perez-Rueda, E., and Collado-Vides, J. (2000). The repertoire of DNA-binding transcriptional regulators in Escherichia coli K-12. Nucleic Acids Res. *28*, 1838-1847.

Rangarajan, A.A., Yilmaz, C., and Schnetz, K. (2022). Deletion of FRT-sites by no-SCAR recombineering in Escherichia coli. Microbiology *168*.

Reinders, A., Hee, C.S., Ozaki, S., Mazur, A., Boehm, A., Schirmer, T., and Jenal, U. (2016). Expression and Genetic Activation of Cyclic Di-GMP-Specific Phosphodiesterases in Escherichia coli. J. Bacteriol. *198*, 448-462.

Richter, A.M., Povolotsky, T.L., Wieler, L.H., and Hengge, R. (2014). Cyclic-di-GMP signalling and biofilm-related properties of the Shiga toxin-producing 2011 German outbreak Escherichia coli O104:H4. EMBO Mol. Med. *6*, 1622-1637.

Römling, U., and Galperin, M.Y. (2017). Discovery of the Second Messenger Cyclic di-GMP. Methods Mol. Biol. *1657*, 1-8.

Römling, U., Galperin, M.Y., and Gomelsky, M. (2013). Cyclic di-GMP: the First 25 Years of a Universal Bacterial Second Messenger. Microbiol. Mol. Biol. Rev. *77*, 1-52.

Römling, U., Gomelsky, M., and Galperin, M.Y. (2005). C-di-GMP: the dawning of a novel bacterial signalling system. Mol. Microbiol. *57*, 629-639.

Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., de Vroom, E., van der Marel, G.A., van Boom, J.H.*, et al.* (1987). Regulation of cellulose synthesis in Acetobacter xylinum by cyclic diguanylic acid. Nature *325*, 279-281.

Santos-Zavaleta, A., Salgado, H., Gama-Castro, S., Sanchez-Perez, M., Gomez-Romero, L., Ledezma-Tejeida, D., Garcia-Sotelo, J.S., Alquicira-Hernandez, K., Muniz-Rascado, L.J., Pena-Loredo, P.*, et al.* (2019). RegulonDB v 10.5: tackling challenges to unify classic and

high throughput knowledge of gene regulation in E. coli K-12. Nucleic acids research *47*, D212-D220.

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*, 4774-4781.

Schuhmacher, J.S., Thormann, K.M., and Bange, G. (2015). How bacteria maintain location and number of flagella? FEMS Microbiol. Rev. *39*, 812-822.

Schumacher, M.A., Zeng, W., Findlay, K.C., Buttner, M.J., Brennan, R.G., and Tschowri, N. (2017). The Streptomyces master regulator BldD binds c-di-GMP sequentially to create a functional BldD2-(c-di-GMP)4 complex. Nucleic Acids Res. *45*, 6923-6933.

Shimada, T., Fujita, N., Maeda, M., and Ishihama, A. (2005). Systematic search for the Cra-binding promoters using genomic SELEX system. Genes Cells *10*, 907-918.

Shimada, T., Hirao, K., Kori, A., Yamamoto, K., and Ishihama, A. (2007). RutR is the uracil/thymine-sensing master regulator of a set of genes for synthesis and degradation of pyrimidines. Mol. Microbiol. *66*, 744-757.

Shimada, T., Ogasawara, H., and Ishihama, A. (2018). Single-target regulators form a minor group of transcription factors in Escherichia coli K-12. Nucleic Acids Res. *46*, 3921-3936.

Steiner, S., Lori, C., Boehm, A., and Jenal, U. (2013). Allosteric activation of exopolysaccharide synthesis through cyclic di-GMP-stimulated protein-protein interaction. EMBO J. *32*, 354-368.

Sundriyal, A., Massa, C., Samoray, D., Zehender, F., Sharpe, T., Jenal, U., and Schirmer, T. (2014). Inherent Regulation of EAL Domain-catalyzed Hydrolysis of Second Messenger Cyclic di-GMP. J. Biol. Chem. *289*, 6978-6990.

Tonthat, N.K., Arold, S.T., Pickering, B.F., Van Dyke, M.W., Liang, S., Lu, Y., Beuria, T.K., Margolin, W., and Schumacher, M.A. (2011). Molecular mechanism by which the nucleoid occlusion factor, SlmA, keeps cytokinesis in check. EMBO J. *30*, 154-164.

Tonthat, N.K., Milam, S.L., Chinnam, N., Whitfill, T., Margolin, W., and Schumacher, M.A. (2013). SlmA forms a higher-order structure on DNA that inhibits cytokinetic Z-ring formation over the nucleoid. Proc. Natl. Acad. Sci. U.S.A. *110*, 10586-10591.

UniProt, C. (2021). UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res. *49*, D480-D489.

Verma, S.C., Qian, Z., and Adhya, S.L. (2019). Architecture of the Escherichia coli nucleoid. PLoS Genet. *15*, e1008456.

Wall, E., Majdalani, N., and Gottesman, S. (2018). The Complex Rcs Regulatory Cascade. Annu. Rev. Microbol. *72*, 111-139.

Wang, X., Montero Llopis, P., and Rudner, D.Z. (2013). Organization and segregation of bacterial chromosomes. Nat. Rev. Genet. *14*, 191-203.

Weber, H., Pesavento, C., Possling, A., Tischendorf, G., and Hengge, R. (2006). Cyclic-di-GMP-mediated signalling within the sigma network of Escherichia coli. Mol. Microbiol. *62*, 1014-1034.

Weinhouse, H., Sapir, S., Amikam, D., Shilo, Y., Volman, G., Ohana, P., and Benziman, M. (1997). c-di-GMP-binding protein, a new factor regulating cellulose synthesis in Acetobacter xylinum. FEBS Lett. *416*, 207-211.

Yilmaz, C., Rangarajan, A.A., and Schnetz, K. (2020). The transcription regulator and c-di-GMP phosphodiesterase PdeL represses motility in Escherichia coli. J. Bacteriol. 203, JB.00427-00420

Yilmaz, C., and Schnetz, K. (2022). High Abundance of Transcription Regulators Compacts the Nucleoid in Escherichia coli. J. Bacteriol *204*, e00026-00022.

Zaritsky, A., and Woldringh, C.L. (2015). Chromosome replication, cell growth, division and shape: a personal perspective. Front. Microbiol. *6*, 756.

Zawadzki, P., Stracy, M., Ginda, K., Zawadzka, K., Lesterlin, C., Kapanidis, A.N., and Sherratt, D.J. (2015). The Localization and Action of Topoisomerase IV in Escherichia coli Chromosome Segregation Is Coordinated by the SMC Complex, MukBEF. Cell rep. *13*, 2587-2596.

Zubay, G., Schwartz, D., and Beckwith, J. (1970). Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Natl. Acad. Sci. U.S.A. *66*, 104-110.

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Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Erklärung zur Datensicherung

Sämtliche Daten, die infolge der Dissertation zugrundeliegenden Arbeiten generiert wurden, sind wie in den Veröffentlichungen beschrieben sowie im Labor internen Netzwerkspeicher der Universität zu Köln gespeichert. Versuchsmaterialien sind in der Labor eigenen Stamm-,Plasmid- und Oligosammlung vorhanden.

Teilpublikationen:

I. Cihan Yilmaz, Aathmaja Anandhi Rangarajan, Karin Schnetz
The Transcription Regulator and c-di-GMP Phosphodiesterase PdeL Represses Motility in *Escherichia coli*

II. Cihan Yilmaz, Karin Schnetz
High Abundance of Transcription Regulators Compacts the Nucleoid in *Escherichia coli*

III. Aathmaja Anandhi Rangarajan, Cihan Yilmaz, Karin Schnetz
Deletion of FRT-sites by no-Scar recombineering in *Escherichia coli*

Köln, den 04.Juli 2022

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