Regulatory mechanisms in the coordination of motility and curli fimbriae-mediated adhesion in *Escherichia coli*

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Abbreviations

αCTDa	carboxy-terminal domain of the α subunit of RNA polymerase
αNTD	amino-terminal domain of the α subunit of RNA polymerase
AMP	adenosine monophosphate
AmpR	ampicillin resistance
APS	ammonium persulfate
A-site	active site
ATP	adenosine triphosphate
β-gal. act.	β-galactosidase activity
BCIP	5-bromo-4-chloro-3-indolyl phosphate
cAMP	cyclic adenosine monophosphate
cat	chloramphenicol resistance gene
c-di-GMP	cyclic di-guanosine monophosphate
CmR	chloramphenicol resistance
CRP	cAMP receptor protein
CTD	carboxy-terminal domain
C-terminal	carboxy-terminal
DGC	diguanylate cyclase
DIG	digoxigenin
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	1,4,-dithiothreitol
EDTA	ethylenediaminetetraacetate
E	RNA polymerase core enzyme
Eσ	RNA polymerase holoenzyme
Fig.	figure
GDP	guanosine diphosphate
GMP	guanosine monophosphate
GTP	guanosine triphosphate
IG	immunoglobulin
IPTG	isopropyl-β-D-1-thiogalactopyranoside
I-site	inhibitory site
kan	kanamycin resistance gene
KanR	kanamycin resistance

LB	Luria-Bertani
mRNA	messenger RNA
NBT	nitroblue tetrazolium chloride
NTD	amino-terminal domain
N-terminal	amino-terminal
OD	optical density
ONPG	ortho-nitrophenyl-β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDE	(c-di-GMP-specific) phosphodiesterase
poly[d(I-C)]	poly-deoxy-inosinic-deoxy-cytidylic acid
PPi	pyrophosphate
(p)ppGpp	guanosine penta- and tetraphosphate
RNA	ribonucleic acid
RNAP	RNA polymerase
SDS	sodium dodecyl sulfate
spec.	specific
tab.	table
TEMED	N,N,N',N'-Tetramethylethylenediamine
TetR	tetracycline resistance
Tn	transposon
Tris	trishydroxyaminomethane
UP-element	upstream regulatory element
wt	wild-type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Summary

Depending on the environmental conditions, bacteria can either exist as single motile cells, also called planktonic cells, or they adhere to each other and to surfaces with the help of adhesive structures, thus becoming sedentary cells that can also form biofilms. In this work the transition between these two lifestyles was investigated in the model organism and gram-negative bacterium *Escherichia coli* that, when cultivated in nutrient-rich medium, switches from the planktonic to the adhesive lifestyle upon entry into the stationary phase of growth. The central aim was the identification and detailed characterization of regulatory processes involved in the coordination of the two major features of the planktonic and the adhesive lifestyle in this organism, i.e. flagella-driven motility and curli fimbriae-mediated adhesion.

The results of these studies demonstrate that the two regulatory cascades directing flagellar motility and curli fimbriae expression communicate with each other through multiple layers of negative "cross-talk", which results in an inverse coordination of the two systems. One of these regulatory links is established by FliZ, a protein under flagellar control that acts as a general inhibitor of the master regulator of the general stress response, σ^S, and therefore also interferes with σ^S-dependent curli fimbriae formation. During the post-exponential growth phase, when flagellar gene expression peaks but σ^S already starts to accumulate in the cell, FliZ temporarily gives priority to motility over the general stress response.

Further analysis of the mechanism that allows FliZ to exert its comprehensive effect on σ^S-dependent gene expression led to the identification of a novel and unprecedented mechanism of interference with sigma factor activity. Detailed *in vivo* and *in vitro* analyses revealed that FliZ does not act as a conventional anti-σ factor, but directly binds to the -10 region of distinct σ^S-dependent promoters with a structural element in FliZ that strongly resembles a promoter recognition element in σ^S and thus interferes with the activity of this sigma factor through mimicry of the promoter recognition mechanism. Furthermore, this work also clarified the role FliZ plays in the regulation of motility in *E. coli*. By providing the first direct molecular mechanism of FliZ action, this study will also help to elucidate the yet unidentified mechanistic details of FliZ-mediated regulation of motility and virulence in other bacterial species.

The FliZ-mediated communication between the motility and curli fimbriae systems is integrated with regulatory links on other levels of the two control cascades that have been identified in the course of this work and in parallel studies performed by the Hengge group. Together these data were combined into a comprehensive model describing the sequence of

events that direct the switch from motility to curli fimbriae-mediated adhesion. Finally, these analyses were complemented by studies revealing several yet unknown details of c-di-GMP-mediated control of curli fimbriae expression that add more detail to this model, but also contribute to our understanding of the general principles of c-di-GMP signalling in bacteria, which is still incomplete.

Zusammenfassung

Je nach Umweltbedingungen existieren Bakterien entweder als einzellige motile Zellen, als so genannte planktonische Zelle, oder aber als sesshafte Zellen, die sich mit Hilfe von adhäsiven Strukturen aneinander und an Oberflächen anheften und dann auch in der Lage sind, Biofilme zu bilden. In der vorliegenden Arbeit wurde der Übergang zwischen diesen beiden Lebensweisen in dem Modellorganismus und gram-negativen Bakterium *Escherichia coli* untersucht, das bei Kultivierung in nährstoffreichem Medium beim Eintritt in die stationäre Wachstumsphase von der planktonischen zur adhäsiven Lebensweise umschaltet. Hauptziel war dabei die Identifizierung und detaillierte Charakterisierung von regulatorischen Prozessen, die an der Koordination der beiden Hauptmerkmale der planktonischen und adhäsiven Lebensweise in diesem Organismus beteiligt sind: der Flagellen-gesteuerten Motilität und der Curli-Fimbrien-vermittelten Adhäsion.

Die Ergebnisse dieser Untersuchungen zeigen, dass die beiden Regulationskaskaden, welche die flagellare Motilität und die Bildung der Curli-Fimbrien kontrollieren, durch negative wechselseitige Einflussnahme auf mehreren Ebenen miteinander kommunizieren, wodurch eine inverse Koordination der beiden Systeme erreicht wird. Eine dieser regulatorischen Verbindungen wird durch das flagellar kontrollierte Protein FliZ vermittelt, das einen allgemeinen inhibitorischen Einfluss auf die Aktivität des Masterregulators der generellen Stressantwort σ^S ausübt und damit auch die σ^S-abhängige Curli-Fimbrien-Bildung reprimiert. In der post-exponentiellen Wachstumsphase, in der die flagellare Genexpression ihren Höhepunkt erreicht, aber auch σ^S schon in der Zelle akkumuliert, gibt FliZ der Motilität vorübergehend den Vorzug gegenüber der generellen Stressantwort.

Eine genauere Analyse der Wirkungsweise, die es FliZ ermöglicht, einen so umfassenden Einfluss auf die σ^S-abhängige Genexpression zu nehmen, führte zur Identifikation eines bislang unbeschriebenen Mechanismus zur Aktivitätsinhibition von Sigmafaktoren. Detaillierte *in-vivo*- und *in-vitro*-Analysen zeigten, dass FliZ nicht als konventioneller Anti-Sigmafaktor wirkt, sondern mit Hilfe eines Strukturelementes, das große Ähnlichkeit zu einem Promotor-Erkennungselement in σ^S aufweist, direkt an die -10-Region bestimmter σ^S-abhängiger Promotoren bindet und so die Aktivität dieses Sigmafaktors durch Mimikry des Promotorerkennungsmechanismus inhibiert. Außerdem konnte im Rahmen dieser Arbeit die Rolle von FliZ in der Motilitätsregulation in *E. coli* aufgeklärt werden. Durch die erstmalige Beschreibung einer direkten molekularen Wirkungsweise von FliZ, kann diese Arbeit auch bei der Aufklärung der bislang unbekannten mechanistischen

Details der FliZ-vermittelten Regulation von Motilität und Virulenz in anderen Bakterienarten helfen.

Die durch FliZ vermittelte Kommunikation zwischen den Motilitäts- und Curli-Fimbrien-Systemen ist mit weiteren regulatorischen Verbindungen auf anderen Ebenen der beiden Regulationskaskaden verschaltet, die in dieser Arbeit und in parallel in der Arbeitsgruppe Hengge durchgeführten Studien identifiziert werden konnten. Diese Daten wurden in einem umfassenden Model zusammengefasst, das die Abfolge der Ereignisse beschreibt, die das Umschalten von Motilität zur Curli-Fimbrien-vermittelten Adhäsion steuern. Schließlich wurden diese Analysen durch die Aufklärung einiger bislang unbekannter Details der c-di-GMP-vermittelten Curli-Fimbrien-Regulation ergänzt, die dieses Model erweitern und außerdem zu unserem noch immer unvollständigen Verständnis genereller Prinzipien c-di-GMP-vermittelter Signaltransduktion beitragen.

1. Introduction

1.1 Regulation of gene expression in bacteria

In their natural environments bacteria are exposed to rapidly changing conditions that require the ability to quickly adapt to a wide variety of stresses such as nutrient limitation, temperature shifts or varying osmolarity. Adaptation of bacteria to harsh conditions involves the induction of systems that counteract the stressor, repair damage inflicted upon cell components and endow the cell with a general resistance against adverse conditions. An appropriate induction of these protective systems in response to different stresses requires complex regulatory mechanisms that result in reprogramming of gene expression in a way that enables the cell to meet the specific requirements. Regulation of gene expression comprises a vast variety of mechanisms targeting all levels of gene expression, i.e. transcription, RNA stability and translation and is complemented by regulation of stability and activity of the gene product. In bacteria, gene regulation mainly takes part at the level of transcription and in the following part I will focus on aspects of transcriptional regulation, mainly at the example of *Escherichia coli*, which served as the most important model organism in the research done in this field.

1.1.1 RNA polymerase structure

RNA polymerase (RNAP), the molecular machine responsible for DNA-dependent RNA synthesis in cellular organisms, shares a common subunit composition of its core element in all domains of life. The catalytically competent core enzyme, a protein complex resembling a crab claw in its shape, has a molecular mass of approximately 400 kDa and is composed of two α subunits, the β an β′ subunits and the ω subunit (Fig. 1.1) (Burgess 1969; Zhang et al. 1999). The two biggest subunits β and β′ constitute the pincers of the claw and confine an internal channel with the active site of the enzyme situated at the base of the channel. In the active site, a Mg^{2+} ion is chelated by residues within a universally conserved sequence motif of the β′ subunit. One of the two α subunits binds primarily to the β subunit while the other one primarily contacts the β′ subunit. The contacts to the β and β′ subunits are made by the respective amino-terminal domain of the α subunits (αNTD), which is connected to the carboxy-terminal domain (αCTD) by a flexible linker. The ω subunit, a small subunit that predominantly binds to the β′ subunit, plays a role as a chaperone of the latter and in the assembly of the core subunits (Mathew and Chatterji 2006).

In order to bind to promoter DNA, the core enzyme needs to associate with the σ subunit to form the RNAP holoenzyme which is capable of initiating transcription (Burgess et al. 1969). In the holoenzyme, the σ subunit is bound predominantly on the core surface through extensive interactions with the core enzyme, the most intensive ones involving the β´ subunit. The σ subunit structure resolved by crystallography identified three flexibly linked domains and an extended linker between the two carboxy-terminal (C-terminal) domains (Murakami et al. 2002b; Vassylyev et al. 2002). The structure of the amino-terminal (N-terminal) region, present in a subgroup of sigma factors (see 1.1.3.1) has not yet been resolved. Association of σ with the core enzyme introduces conformational changes resulting in alterations in sites important for RNAP function.

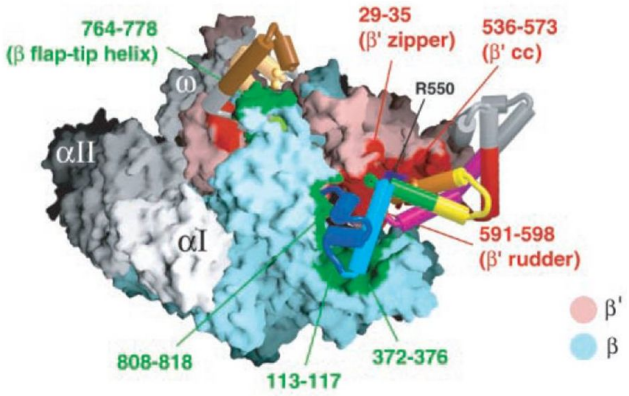

Fig. 1.1: Structure of RNA polymerase holoenzyme. Crystal structure of *Thermus aquaticus* RNA polymerase core enzyme bound to the σ subunit. The core enzyme is shown as a molecular surface with the subunits coloured cyan (β), pink (β´), gray (the two α-CTDs, denoted αI and αII, and ω). The σ subunit is represented by an α-carbon backbone worm with cylinders depicting α helices. Differently coloured cylinders reflect different conserved regions within σ (for information on conserved sigma regions see 1.1.3.1). Green and red areas depict surfaces of β and β´ respectively, that are within 4Å of any σ atoms and the β and β´ residues involved are indicated. A blue patch marks the exposed surface of β´ residue R550 which is important for interaction with σ. (This figure is a reproduction from (Murakami et al. 2002b)).

1.1.2 The transcriptional cycle

Binding of the RNAP holoenzyme to promoter sequences is accompanied by additional changes in holoenzyme conformation and comprises several structural transition intermediates of the RNAP-promoter complex (Murakami et al. 2002a).

In the initial *closed promoter complex* solvent-exposed promoter recognition elements in σ mediate binding to specific promoter elements in the double stranded DNA duplex (for detailed information on promoter recognition see 1.1.3.3). Additional DNA contacts can be mediated by binding of the α subunit C-terminal domains to upstream regulatory sequences (UP-elements) present in certain promoters (Gourse et al. 2000). The sigma subunit then

triggers melting of the DNA duplex finally resulting in the formation of the transcription competent *open promoter complex* in which the DNA duplex is unwound to form the transcription bubble that spans the region containing the transcriptional start site, which is placed in the active site of the enzyme (Murakami et al. 2002a). Upon open complex formation, RNA synthesis is initiated by phosphodiester bond formation between nucleotide substrates bound to the template DNA strand in the active site.

Before switching to a stable elongation conformation, RNAP goes through several cycles of *abortive elongation*. Here, σ contacts with the promoter as well as steric interference of parts of σ with the elongating RNA chain lead to release of short RNA transcripts while RNAP remains bound to the promoter (Carpousis and Gralla 1980; Hsu 2002; Murakami et al. 2002b). Once the RNA transcript reaches the length of about 8-11 nucleotides, RNAP escapes the promoter, and transitions to the productive *elongation complex* conformation (Borukhov and Nudler 2008). While this transition is often accompanied by the release of the σ subunit, σ release is not obligatory and σ can stay bound to the core enzyme throughout the transcription cycle (Bar-Nahum and Nudler 2001). In the elongation complex, RNAP continues RNA synthesis while moving along the DNA until it encounters pausing or termination signals, which trap the enzyme and in the case of termination signals lead to dissociation from DNA. Two important mobile elements of the β´ subunit in the active centre of RNAP, named bridge helix and trigger loop, are thought to play a central role in many important processes at this stage of the transcription process by participating in catalysis, establishment of transcriptional fidelity, forward motion, regulation of elongation speed and termination of transcription (Borukhov and Nudler 2008).

Termination can be either intrinsic or factor-dependent. Intrinsic termination takes place at stable RNA hairpins followed by a stretch of several uridine nucleotides and is based on the inactivation and destabilization of the elongation complex (Gusarov and Nudler 1999). Factor-dependent termination depends on the presence of regulatory factors, the most important one being the protein Rho. The Rho protein assembles to ring-shaped homohexameric complexes that attach to C-rich sites, called *rut* sites on the emerging RNA molecule. Interaction with RNA induces the ATPase activity of Rho that provides the energy for wresting the RNA from RNAP and the DNA-RNA duplex in the transcription bubble (Richardson 2002). Riboswitches represent another prominent example of factor-dependent termination. In riboswitch RNAs, segments of an RNA molecule are employed to directly reflect physiological conditions without the involvement of any other cellular regulator. Changes in temperature or binding of specific ligands that can vary vastly in their nature,

including for instance metal ions, complex cofactors and tRNA molecules, induce structural shifts in the RNA molecule. This in turn promotes structural transitions in RNA elements that affect gene expression, e.g. by changing the accessibility of the translation initiation site (Shine-Dalgarno sequence) or by controlling transcription termination by preventing or inducing the formation of termination helices (Henkin 2008).

1.1.3 Regulation of gene expression by sigma factors

1.1.3.1 Reprogramming of gene expression by alternative sigma factors

Adaptation to changing environmental conditions, but also growth phase and lifestyle transitions (e.g. the transition from exponential growth to stationary phase), which are often accompanied by morphological changes (e.g. expression of adhesive cell surface structures) or differentiation events (e.g. sporulation) require massive reprogramming of gene expression. This is mainly realized by the redirection of RNAP to new sets of genes with the help of alternative sigma factors. Transcription factors can introduce additional alterations of the transcriptional program by activating or repressing certain subsets of genes (see 1.1.4).

Most bacteria possess a primary (or housekeeping/vegetative) sigma factor involved in the transcription of housekeeping genes, and several alternative σ subunits. The number of alternative sigma factors per bacterial genome differs vastly between the species, with species living in more complex environments generally exhibiting higher numbers of sigma factors (Kill et al. 2005). Besides their common function of promoter recognition and melting, sigma factors can be grouped into two families that differ in structure and function (Gruber and Gross 2003; Helmann 2010). Most sigma factors belong to the σ^{70} family based on their sequence similarity to the housekeeping sigma factor σ^{70} in *E. coli*. The smaller family exhibits similarity to the *E. coli* sigma factor σ^{54} and differs from the σ^{70} family in the recognition of differently positioned promoter elements and the need for ATP and ATPases acting as activator proteins for open complex formation (Wigneshweraraj et al. 2008).

The σ^{70} group is further divided into phylogenetic groups and based on sequence conservation between certain groups, four conserved regions, including sub-regions were defined (Fig. 1.2) (Lonetto et al. 1992). Regions 2 and 4, which are involved in recognition of the -10 and -35 promoter elements (see 1.1.3.3), are present in all groups. The first group comprises σ^{70} orthologs serving as indispensible housekeeping sigma factors in all bacteria. The housekeeping sigma factors fold into three stable flexibly linked domains (σ_2-σ_4), with an extended linker between the two C-terminal domains, and an N-terminal, poorly conserved acidic region (region 1.1), the structure of which has not been determined yet (Campbell et al.

2002; Murakami et al. 2002b; Vassylyev et al. 2002). Members of group 2 are sigma factors that share high sequence similarity to group 1 sigma factors but are not essential for growth under normal conditions. The master regulator of the general stress response in *E. coli*, σ^S, is a member of this group (Hengge 2010a). The flagellar sigma factor σ^{28} (FliA) (Chilcott and Hughes 2000) and the heat shock sigma factor σ^{32} (Lim and Gross 2010) from *E. coli*, as well as the master regulator of the general stress response in *Bacillus subtilis*, σ^B (Hecker et al. 2007), are examples of the more divergent group 3 sigma factors, which do not have conserved region 1. Group 4 sigma factors only display the two conserved regions involved in promoter recognition (region 2 and 4). Due to the participation of many group 4 members in stress responses associated with the cell envelope this group of sigma factors is also known as extracytoplasmic function (ECF) sigma factors. The regulator of the envelope stress response in *E. coli* σ^E (Ades et al. 2010) and σ^{FecI}, an *E. coli* sigma factor involved in iron transport (Braun and Mahren 2005), belong to this diverse group. Further groups have recently been suggested based on the discovery of proteins acting as sigma factors but showing only faint sequence similarity with the other groups (Helmann 2010).

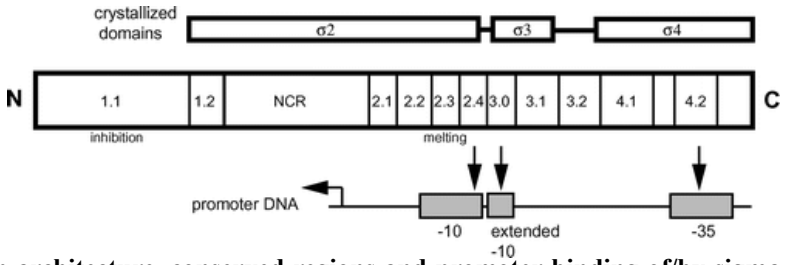

Fig. 1.2: Domain architecture, conserved regions and promoter binding of/by sigma factors. Based on the *E. coli* sigma factor σ^{70}, the four conserved regions, including sub-regions and a non-conserved region (NCR), as well as the domains for which structural information is available are indicated. Functional roles of certain regions in inhibition of free sigma factor binding to DNA (Dombroski et al. 1992; Camarero et al. 2002) and in promoter interactions are noted and illustrated in more detail for recognition of promoter elements. (This figure is a reproduction form (Gruber and Gross 2003)).

The six alternative sigma factors encoded in the *E. coli* genome activate distinct regulons of varying size, ranging from 10% of all genes in the case of σ^S (Weber et al. 2005) to a single operon as observed for σ^{FecI} (Braun and Mahren 2005). Details on the function and role of two alternative sigma factors relevant for the work presented here, i.e. σ^S and σ^{28}, will be discussed in later sections (see 1.1.3.4. and 1.3.3.2).

1.1.3.2 Regulation of alternative sigma factor activity

Alternative sigma factors are tightly controlled and activate their respective regulons only under specific physiological conditions. How efficient a sigma factor is in recruiting RNAP

holoenzyme to the genes of its regulon and in initiating transcription depends on the cellular level of the sigma factor, its availability for binding to RNAP core enzyme, its ability to compete with other sigma factors for binding to RNAP core enzyme and on the activity of the resulting holoenzyme. All of these factors are subject to regulation and contribute to successful induction of alternative sigma factor regulons under appropriate conditions, despite the fact that in *E. coli* the housekeeping sigma factor was shown to be the most abundant sigma factor (Grigorova et al. 2006) and to have the highest affinity for core enzyme (Maeda et al. 2000).

Sigma factor concentrations are often controlled by regulation on all levels of synthesis and by stability control, as seen for example in the induction of the general stress response by σ^S in *E. coli* (see 1.1.3.4; (Hengge-Aronis 2002a; Hengge 2010a)).

The availability of a sigma factor for binding to RNAP core enzyme and its activity can be significantly reduced by antagonists such as anti-sigma factors. Typically, an anti-sigma factor binds to its cognate sigma factor and inhibits its activity through occlusion of binding sites and other conformational constraints that prevent holoenzyme formation and promoter binding (Campbell et al. 2008). Regulation of the activity of anti-sigma factors depends on versatile and often highly complex mechanisms that can imply reversible and irreversible modifications of the anti-sigma factor molecules, with the former ones probably allowing faster and more energy efficient responses (Helmann 2010). Proteolysis of several transmembrane anti-sigma factors that antagonize group 4 sigma factors in response to adequate signals is a prominent example for irreversible modification of an anti-sigma factor that triggers release of the sigma factor (Heinrich and Wiegert 2009). Moreover, secretion of FlgM, the antagonist of the flagellar sigma factor σ^{28} in *Escherichia coli* is used by the cell to monitor the progression of flagellar assembly and couple flagellar gene expression to assembly of the flagellum (see 1.3.3.2; (Chilcott and Hughes 2000)). In contrast, reversible phosphorylation plays a role in the regulation of the *B. subtilis* general stress response sigma factor σ^B. σ^B is inactivated by binding of the anti-sigma factor RsbW. Release of σ^B upon stress is induced by "partner-switching" of the anti-sigma factor, which then binds to the anti-anti-sigma factor RsbV. Sequestration of the anti-sigma factor by its anti-anti-sigma factor is only possible if the latter is not phosphorylated. Phosphorylation of RsbV is mediated by the anti-sigma factor RsbW, but stress conditions lead to dephosphorylation of RsbV by specific phosphatases, thereby ultimately triggering the release of σ^B (Hecker et al. 2007). Interestingly, the response regulator RssB, which targets σ^S for ClpXP-mediated proteolysis upon phosphorylation of its receiver domain, was shown to be able to act as an anti-σ^S-factor

under certain conditions *in vivo*. This lead to the speculation that RssB may originally have been an anti-sigma factor that was recruited as a σ^S-specific targeting factor for ClpXP-mediated proteolysis during evolution (Becker et al. 2000).

An alternative way of influencing sigma factor competition for core enzyme is represented by the *E. coli* protein Crl, a factor that specifically helps σ^S in its competition for core enzyme (Typas et al. 2007a). Moreover, factors negatively affecting the efficiency of holoenzyme containing the housekeeping sigma factor passively support the activity of holoenzymes associated with alternative sigma factors. Examples are the second messenger (p)ppGpp, which together with the DksA protein strongly decreases $E\sigma^{70}$-driven ribosomal RNA synthesis, thereby increasing the pool of available core enzyme (see 1.2.1; (Srivatsan and Wang 2008)), the conserved bacterial non-coding 6S RNA, which mimics an open promoter complex that traps $E\sigma^{70}$, but no alternative holoenzymes (Trotochaud and Wassarman 2005) and the Rsd protein, which acts as an anti-σ^{70} factor (Jishage and Ishihama 1998). Induction of these factors in stationary phase or under stress conditions along with accumulation of σ^S paves the way for efficient induction of the σ^S regulon. Similarly, certain other factors that reflect the physiological state of the cell are known to differentially influence the activity of $E\sigma^{70}$ and $E\sigma^S$. Relaxation of chromosomal supercoiling in stationary phase and accumulation of potassium glutamate during hyperosmotic stress positively affect $E\sigma^S$ but not $E\sigma^{70}$-mediated transcription, thereby providing $E\sigma^S$ with a selective advantage over $E\sigma^{70}$ under conditions requiring the induction of the σ^S regulon (Ding et al. 1995; Kusano et al. 1996; Bordes et al. 2003; Lee and Gralla 2004).

1.1.3.3 Promoter recognition and discrimination by sigma factors

Members of the σ^{70} family bind to hexameric promoter recognition elements located around positions -35 and -10 relative to the transcriptional start site (Fig. 1.2). Residues within region 2.4 of σ make sequence specific contacts with the -10 element, while residues within region 3.0 recognize nucleotides upstream of the -10 element (the extended -10 element) and the two alpha helices containing these residues form a V-shaped structure near the entrance of the active site cleft of RNAP (Barne et al. 1997; Murakami et al. 2002a; Vassylyev et al. 2002). The -35 element is recognized by residues of a helix-turn-helix motif within region 4.2 (Campbell et al. 2002).

Differences in promoter specificity between sigma factors are based on differences in these promoter recognition elements. Alternative sigma factors therefore recognize consensus sequences different from the -10 and -35 consensus sequences recognized by the

housekeeping sigma factor. However, group 1 and 2 sigma factors, including the *E. coli* housekeeping sigma factor σ^{70} and stress sigma factor σ^{S}, respectively, share high sequence similarity, which is reflected in the recognition of very similar promoter sequences. The question how these two sigma factors differentially recognize almost identical promoter sequences when initiating transcription of their distinct operons has puzzled scientists for years. Certain promoter sequence elements as well as co-regulating transcriptional regulators are now known to determine σ^{S} selectivity, i.e. preferred recognition of a promoter by $E\sigma^{S}$ in *E. coli* (Fig. 1.3A, (Hengge-Aronis 2002b; Typas et al. 2007b)). Amongst the promoter elements known to contribute to σ^{S} selectivity, the most important one is a cytosine at position -13 with respect to the transcriptional start site (C(-13)), which is conserved in many σ^{S}-dependent promoters, while σ^{70}-dependent promoters show a bias for guanine at this position (Becker and Hengge-Aronis 2001; Gaal et al. 2001; Weber et al. 2005). A thymine at the neighbouring position -14 also contributes to σ^{S} selectivity, although to a lesser extent (Becker and Hengge-Aronis 2001). The C(-13) (and possibly also the adjacent nucleotide at position -14) is directly contacted by a lysine (K173) located in an α-helix in region 3.0 of σ^{S} that contacts the extended -10 promoter element (Becker and Hengge-Aronis 2001). σ^{70} carries a glutamate residue at the corresponding position (E458). Thus, opposite charges at position 173 and 458 in σ^{S} and σ^{70} respectively account for the different preferences of the two sigma factors for the nucleotide at position -13 (Fig 1.3B). Interestingly, K173 also represents the amino acid bound by the proteolysis recognition factor RssB that targets σ^{S} to ClpXP-mediated degradation (see 1.1.3.4) (Becker et al. 1999).

Many other features can contribute to establishing σ^{S} selectivity of a promoter (summarized in Fig. 1.3A). These are often based on the greater tolerance of σ^{S}-containing holoenzyme ($E\sigma^{S}$) for deviations from both the consensus sequence of the -10 and -35 promoter elements (with some σ^{S}-dependent promoters showing no recognizable -35 element at all) and from the optimal length (17 nucleotides) of the spacer between them (Wise et al. 1996; Bordes et al. 2000; Gaal et al. 2001; Lee and Gralla 2001; Lacour et al. 2003; Typas and Hengge 2006). In contrast, similar deviations impair σ^{70}-containing holoenzyme ($E\sigma^{70}$) more strongly in its activity. In addition, A/T-rich sequences immediately downstream of the -10 region (termed A/T rich discriminator) were shown to increase σ^{S} selectivity, presumably by conveying optimized promoter melting (Ojangu et al. 2000; Lee and Gralla 2001; Pruteanu and Hengge-Aronis 2002; Weber et al. 2005).

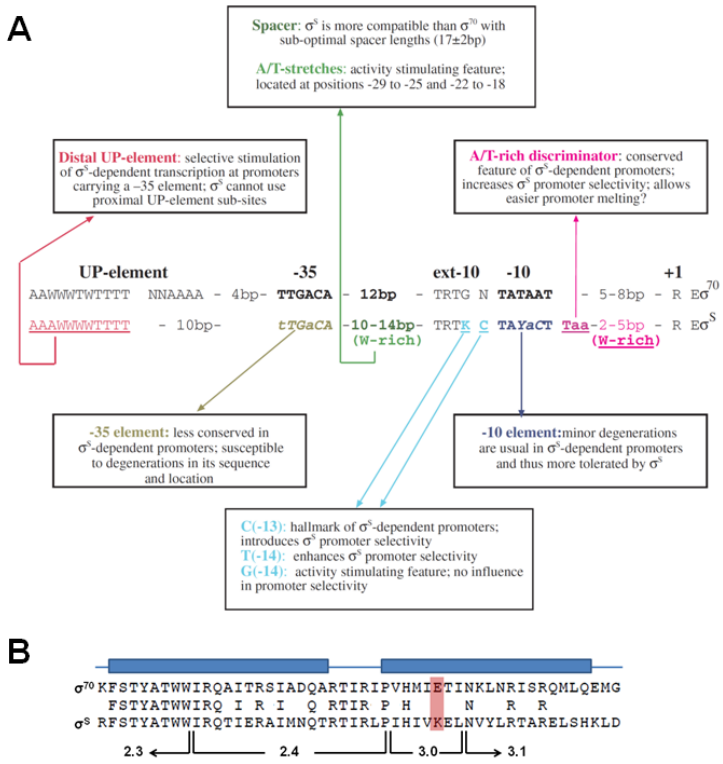

Fig. 1.3: Differential promoter recognition by Eσ^{70} and Eσ^{S}. **(A)** Consensus sequences of σ^{70}- and σ^{S}-dependent promoters and summary of sequence elements contributing to σ^{S} selectivity of a promoter. Bold letters denote elements conserved in most σ^{70}- and σ^{S}-dependent promoters, while positions frequently degenerate in σ^{S}-dependent promoters are printed in italics, with the least conserved ones indicated by lower case letters. Elements other than degenerate -10 and -35 elements that contribute to promoter selectivity are underlined and their functions are summarized in text boxes. In addition, elements that stimulate promoter activity without affecting promoter selectivity are noted. Nucleotide symbols are used as follows: R and Y stands for a purine (A/G) and pyrimidine (T/C) base, respectively, K denotes T or G, W represents A or T and N denotes any of the four nucleotides. (Figure A is a reproduction from (Typas et al. 2007b)). **(B)** Partial alignment between regions in σ^{70} (residues 426 to 475) and σ^{S} (residues 141 to 190) involved in differential promoter recognition. Blue bars denote the last α helix of σ domain 2 an the first α helix of σ domain 3, that contain residues of conserved regions 2.3 to 3.1, which are indicated underneath the alignment. Identical residues in both proteins are noted by the sequence between the two protein sequences. Residues K173 of σ^{S} and E458 of σ^{70} which are involved in differential recognition of the C(-13) in many σ^{S}-dependent promoters are indicated by red background. Location of the α helices is based on the structure of σ^{70} of *Thermus thermophilus* reported by Vassylyev et al. (Vassylyev et al. 2002).

The different ability of Eσ^{70} and Eσ^{S} to utilize UP-elements at promoters with -35 regions generates another feature establishing σ^{S} selectivity of a promoter (Typas and Hengge 2005). UP-elements are AT-rich sequences right upstream of the -35 element that consist of a proximal and a distal (with respect to the -35 element) half-site, each of which can be bound by one α-CTD of RNAP, usually resulting in stimulation of transcription (Gourse et al. 2000). σ^{S} selectivity is induced by the presence of only a distal UP-element half-site, while this scenario impedes Eσ^{70}-mediated transcription, which on the other hand is selected for in the presence of proximal half-sites or whole UP-elements. This difference in UP-element utilization is due to an oppositely charged protein surface patch on σ^{S} and σ^{70}, which enables

σ^{70}, but probably not σ^S to interact with an α-CTD bound to a proximal UP-element half-site (Typas and Hengge 2005).

In addition to promoter elements, a higher susceptibility of $E\sigma^{70}$-mediated transcription to pausing during transcriptional elongation at DNA sites that resemble -10 elements seems to contribute to establish σ^S selectivity of a promoter (Typas et al. 2007b). Finally, certain transcriptional regulators and histone-like proteins, like H-NS (see 1.1.4.3) can determine and alter sigma factor selectivity of a promoter by differentially affecting the activity of $E\sigma^{70}$ and $E\sigma^S$ at a specific promoter (Hengge-Aronis 1999; Typas et al. 2007b; Hengge 2010a). Many σ^S-dependent promoters combine various of these σ^S selectivity-inducing features in a modular fashion, resulting in varying degrees of σ^S dependence (Typas et al. 2007b).

1.1.3.4 σ^S (RpoS) and the general stress response in *E. coli*

Transition into stationary phase and a diverse set of stressful conditions induce the σ^S-mediated general stress response. Signals triggering this response can be integrated at the levels of the above mentioned factors affecting $E\sigma^S$ activity. However, accumulation of σ^S is a prerequisite for successful induction of the σ^S regulon and many stresses therefore affect the synthesis and stability of σ^S. Accumulation of σ^S during transition into stationary phase, but also in response to certain other stresses, e.g. high osmolarity, is based on a combination of mechanisms affecting transcription and translation. This involves the activity of a vast variety of regulators such as (p)ppGpp, small regulatory RNAs and RNA-binding proteins (Hengge-Aronis 2002a; Hengge 2010a). Regulation of σ^S proteolysis plays its most important role in the fast accumulation of σ^S in response to sudden, potentially life-threatening stresses, reflecting its potential for fast responses (Hengge 2009b; Hengge 2010a)). Interestingly, the σ^S element that recognizes the typical C(-13) residue in many σ^S-dependent promoters (see 1.1.3.3) also plays a crucial role in σ^S proteolysis. K173 is the most essential constituent of a turnover element in σ^S that is bound by phosphorylated RssB (Becker et al. 1999). This contact induces a conformational change in σ^S, which exposes a binding site for the ClpX chaperone and thereby initiates ClpXP-mediated proteolysis (Stüdemann et al. 2003).

Accumulation of σ^S triggers σ^S-dependent gene expression. The σ^S regulon comprises almost 500 genes, i.e. 10% of all genes in the *E. coli* genome, with a core set of 140 genes that are most likely induced as a direct consequence of σ^S accumulation, while the remaining genes require additional regulation for induction (Weber et al. 2005). Many σ^S-dependent genes are involved in counteracting the deleterious effects of specific stresses and in addition, induction of σ^S-dependent gene expression generates a broader stress resistance, also against

stressful conditions not directly encountered. Moreover, σ^S dependence of many metabolic enzymes and membrane proteins, including transport systems, reflects the shift of membrane traffic and central metabolic activities in a direction that ensures maintenance rather than growth in cells entering stationary phase or being exposed to adverse conditions. Another large group of σ^S-dependent genes encodes proteins involved in signal transduction and regulation of other processes. Thus, the σ^S regulon represents a regulatory network comprising many hierarchically arranged modules consisting of σ^S-dependent regulators and their target functions (Weber et al. 2005). Interestingly, several σ^S-dependent genes specify biofilm-associated functions, with several important biofilm regulators showing σ^S-dependent induction upon entry into stationary phase. This also includes the expression of adhesive curli fimbriae, which is controlled by a cascade comprising several σ^S-dependent regulators (see 1.3.2).

1.1.4 Regulation of transcription by transcription factors

In addition to transcriptional regulation by alternative sigma factors, additional signals can be integrated through DNA-binding regulators that activate or repress transcription. Control of cellular concentrations and activities of these regulators, for instance by regulated proteolysis, or by altering the DNA binding affinity, e.g. through binding of small ligands or phosphorylation, vastly increases the potential of the cell to modulate gene expression in response to environmental and cellular cues. The mechanisms by which transcriptional regulators modify the activity of the transcriptional machinery are diverse, but several conserved principles can be observed for both activation and repression and the most common ones will be summarized next.

1.1.4.1 Transcriptional activation

Simple activation takes place at promoters that require a single activator bound to a specific sequence, called operator site, to induce transcription. According to the position of the operator sequence and the nature of the interaction between the activator and RNAP, class I and class II activation mechanisms are distinguished (Browning and Busby 2004). In class I activation an activator dimer binds upstream of the -35 promoter element, contacts the α-CTD and thereby recruits RNAP to the promoter. In contrast, class II activators bind to DNA-sites overlapping with the -35 element and interact with domain 4 of the RNAP sigma factor, with possible additional contacts to the α subunits. Class II activators can help to induce

transcription both by recruiting RNAP and by increasing the efficiency of other steps in transcription initiation. In accordance with the differences in binding and interaction with RNAP, class I and II activator binding sites differ in their location. While class I regulator binding sites can be located at varying distances upstream of the core promoter, restricted only by the need for binding of RNAP and the activator on the same face of the DNA double helix, class II activators usually bind centred at promoter positions -41/-42. Activation of the *lac* promoter by CRP, the cyclic AMP receptor protein, bound to a site centred at 61.5 is an example of class I activation, while activation of the *galP1* promoter by CRP bound to a position centred at -41.5 represents a typical class II activation (Busby and Ebright 1999; Lawson et al. 2004). Thus, transcriptional activators are not necessarily restricted to one mode of activation.

A different mechanism of activation involves the introduction of conformational changes in promoter DNA that allow productive promoter binding by RNAP. The MerR family of transcriptional regulators, which includes e.g. the *E. coli* protein SoxR, a regulator involved in the response to oxidative stress and MlrA, a main regulator in the expression of adhesive curli fimbriae (see 1.3.2), employ this mechanism to activate transcription (Brown et al. 2003). Dimers of these regulators usually bind to operator sites within the spacer between the -10 and -35 elements, which has a suboptimal length for recognition by RNAP. Upon modification of the activator in response to environmental signals, e.g. through binding of small ligands, such as metal ions or antibiotics, or by other modifications, the activator protein changes its conformation. This in turn leads to distortion of the operator DNA resulting in repositioning of the -10 and -35 promoter elements in a way that optimizes the distance between them for recognition by the RNAP sigma factor.

In addition to the mechanisms described above, some more unconventional mechanisms of transcriptional activation involve e.g. regulators for which transcriptional activation does not rely on initial DNA binding (Beck et al. 2007). One example is given by the *B. subtilis* regulator Spx, a major regulator in the response to oxidative stress that can act as both a transcriptional activator and a repressor by interacting with the α-CTD of RNAP (Zuber 2004; Zuber 2009). Under oxidative stress, Spx undergoes a conformational change through the formation of an intramolecular disulphide bond that most likely results in productive interaction of the Spx-RNAP complex with the promoter, thus activating transcription (Nakano et al. 2005; Nakano et al. 2010). Also the closely related *E. coli* regulators MarA, SoxS and Rob bind to the α-CTD of RNAP and the activator-RNAP complexes are suggested

to have higher affinity for promoter DNA than the single components (Martin et al. 2002; Dangi et al. 2004).

1.1.4.2 Transcriptional repression

Simple repression, i.e. transcriptional repression involving only a single regulator also shows some common mechanistic features (Browning and Busby 2004). Most transcriptional repressors function by binding to operator sites that overlap with core promoter elements, thereby usually interfering with RNAP binding to these elements. Other proteins repress transcription by looping of DNA sites that carry core promoter elements. This is achieved by the binding of usually multiple repressor molecules at operator sites further upstream and downstream of the core promoter. The AraC protein, that acts both as an activator and repressor of the genes required for arabinose transport and catabolism in *E. coli* uses this mechanism of repression. In the absence of arabinose an AraC dimer binds to two operator half sites separated by 210 nucleotides, resulting in formation of a DNA loop that sterically hinders RNAP binding to two promoters (Schleif 2003). Direct interaction with an activator and interference with its activity is another mechanism employed by some transcriptional regulators, such as CytR, a regulator involved in transport and utilization of ribonucleosides and deoxyribonucleosides in *E. coli*. CytR binding to CRP, which on its own acts as an activator of the *deoP2* promoter, leads to the formation of an unproductive nucleoprotein complex (Shin et al. 2001).

Analogous to the scenarios described for activation, more uncommon modes of repression have been observed. An illustrative example is repression mediated by the Spx protein. In the case of Spx-mediated repression, interaction of Spx with the α-CTD of RNAP interferes with productive interactions between RNAP and activator proteins at certain promoters (Nakano et al. 2003).

Naturally, many promoters are regulated by more than one protein, thereby allowing for the integration of a multitude of signals. Expression from these promoters is the output of complex combinations of several activating and/or repressing mechanisms, in which the regulators involved may either act independently of each other or cooperate (Browning and Busby 2004). Examples of such complex promoters are the promoter controlling the expression of the flagellar master regulator operon *flhDC* and the promoter of the *csgD* gene, which encodes a major regulator of curli fimbriae expression (see 1.3.2 and 1.3.3.3).

1.1.4.3 Nucleoid-associated proteins

A group of proteins called nucleoid-associated proteins that abundantly bind to the chromosome and affect both its global structure and the local DNA conformation in certain areas significantly add to the complexity of transcriptional regulation. Nucleoid-associated proteins, e.g. the *E. coli* proteins integration host factor (IHF), histone-like-nucleotide-structuring protein (H-NS) and DNA-binding protein from starved cells (Dps), globally affect gene expression. Effects on transcription mainly rely on the ability of nucleoid-associated proteins to alter DNA structure, but some of the more direct mechanisms for positive and negative regulation, which have been described above, are used as well by certain nucleoid-associated proteins (McLeod and Johnson 2001).

H-NS binds to intrinsically curved, AT-rich DNA segments. H-NS-mediated repression of many promoters involves the assembly of oligomeric nucleoprotein complexes (Dorman 2004; Fang and Rimsky 2008). These can consist of H-NS complexes that bind to and bridge promoter-flanking DNA sites thereby trapping RNAP, like in the case of the *rrnB P1* promoter involved in rRNA synthesis (Dame et al. 2002). H-NS also represses promoters by binding to sites further upstream or downstream of the core promoter, followed by successive nucleation along the DNA up to the core promoter elements that results in transcriptional silencing, e.g. of the *proU* operon, which encodes a transport system for the osmoprotectant glycine betaine (Lucht et al. 1994). So far, there has not been any unequivocal evidence for direct transcriptional activation by H-NS (Dorman 2004). Rather, positive regulation by H-NS seems to be indirect, for example through H-NS-mediated repression of a transcriptional repressor. Interestingly, H-NS was also demonstrated to selectively interfere with $E\sigma^{70}$-mediated transcription while allowing for transcription by $E\sigma^{S}$, thereby influencing sigma factor selectivity of transcription (see 1.1.3.3) at some promoters (Arnqvist et al. 1994; Shin et al. 2005). This selective repression of $E\sigma^{70}$-activity is based on assembly of an inhibitory nucleoprotein complex around promoter-bound $E\sigma^{70}$, but not $E\sigma^{S}$ (Shin et al. 2005).

Additional complexity is added to these manifold effects that H-NS and other nucleoid-associated proteins can exert on transcription by complex patterns of antagonistic or cooperative co-regulation with other nucleoid-associated proteins and with gene-specific transcription factors (McLeod and Johnson 2001; Fang and Rimsky 2008). In addition, dynamic changes in the pool of nucleoid-associated proteins throughout the growth cycle are likely to mediate global changes in DNA topology and gene expression in a growth phase-dependent fashion (Ali Azam et al. 1999).

1.2 Regulation by nucleotide-based second messengers in bacteria

In all domains of life nucleotide-based second messengers are part of the signal transduction cascades that translate sensed changes in the environment or in intracellular conditions into appropriate cellular responses, such as reprogramming of gene expression. A plethora of nucleotides is involved in the regulation of virtually all aspects of life in animal, plant, and prokaryotic cells.

In bacteria, cyclic adenosine monophosphate (cAMP), guanosine penta- and tetraphosphate ((p)ppGpp) and cyclic di-guanosine monophosphate (c-di-GMP) are amongst the most comprehensively studied nucleotide-based second messengers and since they are also involved in regulation of the phenotypes investigated in this work, the following sections will focus on introducing these three second messengers. However, this list is far from being complete, as it is missing nucleotides such as cGMP, the presence of which in the bacterial world has long been controversial, but could recently be established with the identification, and characterization of guanylyl cyclases from the cyanobacterium *Synechocystis* PCC6803 (Ochoa De Alda et al. 2000; Rauch et al. 2008) and the α-proteobacterium *Rhodospirillum centenum* (Marden et al. 2011). In the latter, cGMP signalling was shown to control the formation of metabolically dormant cysts. In addition, the recent discovery of cyclic di-adenosine monophosphate (c-di-AMP) in *Bacillus subtilis*, with the characterizations of the proteins DisA and YybT as c-di-AMP synthesizing and degrading enzymes, respectively, adds another nucleotide-based signalling molecule to the list (Witte et al. 2008; Rao et al. 2010). Diadenylate cyclase activity of the DNA integrity-scanning protein DisA is suppressed by binding to branched nucleic acids, e.g. stalled replication forks or recombination intermediates, suggesting a role for DisA in the detection of chromosomal DNA damage (Witte et al. 2008). Moreover, a recent report suggests that c-di-AMP is exported from *Listeria monocytogenes* cells through multidrug efflux pumps and in turn triggers the host immune response to this intracellular pathogen (Woodward et al. 2010).

1.2.1 Architecture of second messenger control modules and common principles of signalling

Despite the diversity in the nature of these nucleotides, as well as of the organisms that employ them and the processes regulated by them, a common principle underlies all nucleotide-based second messenger signalling: Two distinct enzymatic activities catalyze the

synthesis and break-down of the second messenger that is able to bind to an effector molecule as an allosteric regulator. In turn, the effector interacts with a molecular target that is part of the output function regulated by the second messenger (Fig. 1.4).

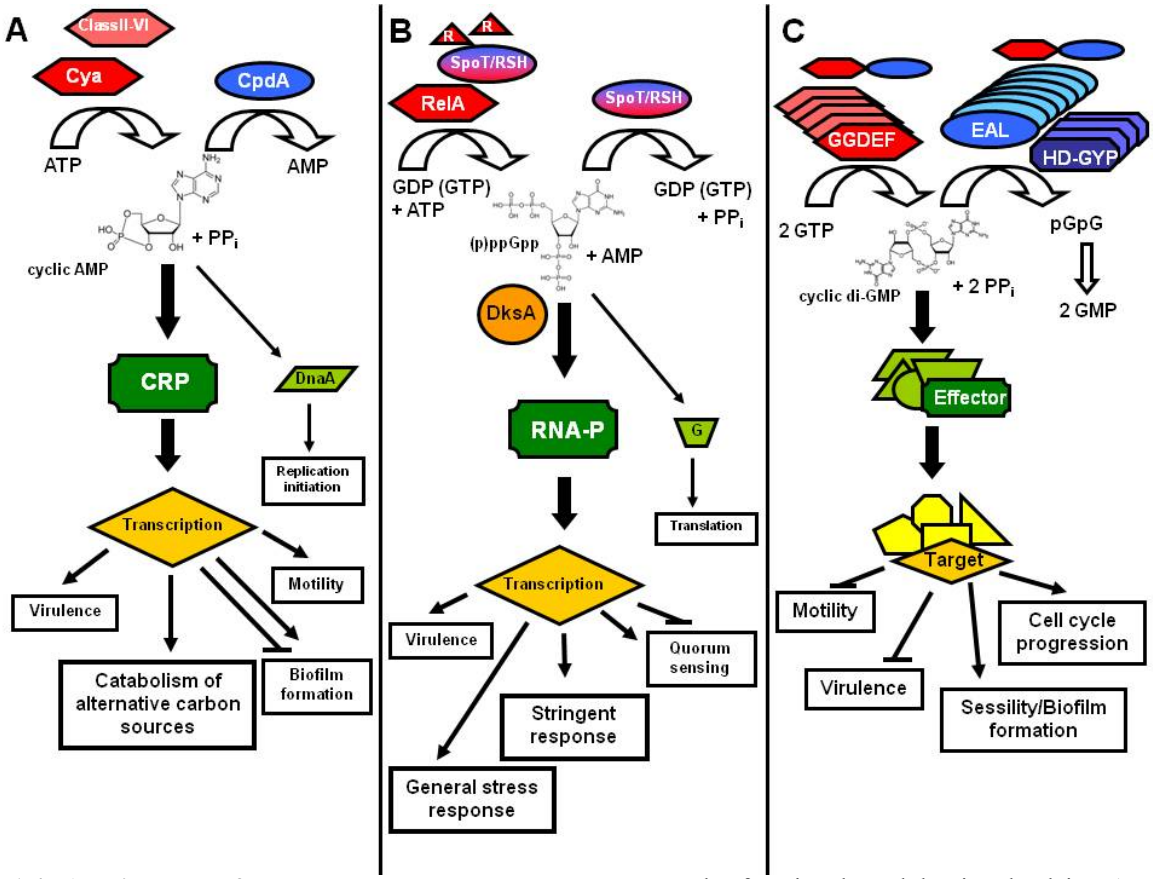

Fig. 1.4: Architecture of second messenger control modules. The functional modules involved in cAMP signalling **(A)**, (p)ppGpp signalling **(B)** and c-di-GMP signalling **(C)** are compared. Proteins and protein domains that synthesize the respective second messenger are coloured red, while degrading activities are indicated by blue colour. Effector molecules are printed in green and target molecules in yellow. DksA is coloured orange. The replication initiation protein DnaA is also included in (A), since cAMP binding to DnaA has been shown, promoting rapid reactivation of inactive ADP-bound DnaA by removal of bound ADP (Hughes et al. 1988). (p)ppGpp has also been found to bind to certain guanine nucleotide binding proteins (G-proteins, indicated here by a G) (Buglino et al. 2002; Milon et al. 2006), which may constitute additional effectors and in the case of IF2, this has an influence on translation (Milon et al. 2006). R marks small protein fragments, with homology to RelA. A selection of the most important target processes is noted in white boxes. (This figure is a reproduction from (Pesavento and Hengge 2009) with minor changes).

Although all three systems generally function in an analogous way, reflecting the general principles of second-messenger signalling, the cAMP-, (p)ppGpp- and c-di-GMP-based systems show significant differences in the multiplicity of the components that form the second messenger control modules, i.e. in how many synthesizing, degrading, effector and target components exist in parallel in the cell. This is illustrated in figure 1.4, which compares the architecture of the three signalling systems, as well as the cellular processes affected by the respective second messenger, which in many cases overlap between the three systems. The

multiplicity of components allows the definition of functional modules, which are the group of enzymes, effectors and targets that, via a common pool of a second messenger, affect a common functional output.

In *E. coli* and most other γ-proteobacteria bacteria, cAMP is synthesized by a single adenylate cyclase (Cya) in response to carbon limitation, sensed through the absence of sugar transport by the phosphoenol-pyruvate-dependent sugar phosphotransferase system (PTS) and degraded by a single phosphodiesterase (CpdA) (Botsford and Harman 1992; Imamura et al. 1996; Görke and Stülke 2008) (Fig. 1.4A). cAMP binds to the cAMP receptor protein (CRP) and induces conformational changes that render the cAMP-CRP complex capable of binding to specific target DNA sequences. cAMP-CRP functions as both a positive and a negative transcriptional regulator. The result is an induction of enzyme systems specific for catabolism of alternative carbon sources (Botsford and Harman 1992). In addition to inducing the carbon scavenging response, cAMP directly and indirectly regulates a wide range of other cellular functions, such as e.g. flagellum biosynthesis (see 1.3.3.3), biofilm formation and virulence (Botsford and Harman 1992; Jackson et al. 2002; Liang et al. 2007; Görke and Stülke 2008) and the number of known cAMP-regulated genes has constantly increased with the use of whole-genome approaches to identify cAMP target genes ((Hollands et al. 2007) and references cited therein). Other bacterial phyla also express other classes of adenylate cyclases, e.g. class II adenylate cyclases comprising several secreted adenylate cyclases, functioning as toxins and the universal class III cyclases, also present in eukaryotic cells. Different classes of adenylate cyclases can coexist in single bacterial genomes (Baker and Kelly 2004; Shenroy and Visweswariah 2004).

(p)ppGpp signalling is induced in response to signals resulting from nutrient limitations. In β- and γ-proteobacteria, the synthesis and degradation of (p)ppGpp is controlled by two proteins, homologues of the *E. coli* RelA and SpoT proteins. However, other bacteria possess a single RelA/SpoT homolog (RSH proteins for Rel Spo homolog), in some cases together with small fragments with RelA homology, which are also able to synthesize (p)ppGpp (Braeken et al. 2006; Potrykus and Cashel 2008; Srivatsan and Wang 2008) (Fig. 1.4B). In *E. coli* the ribosome-associated RelA protein synthesizes (p)ppGpp in response to amino acid starvation sensed by the presence of uncharged tRNA molecules in the active site of the ribosome. In the bifuctional protein SpoT and in the single RSH proteins present in many other species, the balance between (p)ppGpp synthesis and hydrolysis is altered in response to different sources of stress and nutrient limitation (Potrykus and Cashel 2008). (p)ppGpp binds RNA polymerase as its direct effector leading to global changes in the transcription pattern of

the cell and the DksA protein is involved in (p)ppGpp signalling as a factor potentiating (p)ppGpp-mediated effects (Magnusson et al. 2005; Potrykus and Cashel 2008; Srivatsan and Wang 2008). The effects of an increase in the intracellular (p)ppGpp concentration exceed those of the classical stringent response characterized by a decrease in stable RNA synthesis, inhibition of growth and reorganization of the metabolism and resource allocation in the cell. (p)ppGpp is involved in the regulation of many other bacterial functions including virulence and quorum sensing (Braeken et al. 2006; Potrykus and Cashel 2008). As (p)ppGpp positively controls the expression and activity of σ^S, the general stress response also depends on (p)ppGpp (Hengge-Aronis 2002a; Jishage et al. 2002; Traxler et al. 2006; Traxler et al. 2008).

In comparison to the two systems described above, the multiplicity of synthesizing and degrading proteins, as well as effector molecules in many bacterial species is particularly striking in c-di-GMP-signalling, as will be detailed in the following section.

1.2.2 c-di-GMP signalling

1.2.2.1 Protein domains involved in c-di-GMP signalling

The three protein domains involved in turnover of c-di-GMP are named after the most prominent conserved amino acid motifs found in their active sites.

Formation of c-di-GMP is mediated by diguanylate cyclases (DGCs) and requires dimerization of two domains carrying an intact GGDEF (or GGEEF) sequence motif in the active site (A-site) of the domain (Paul et al. 2004; Malone et al. 2007). c-di-GMP is synthesized from GTP by a mechanism closely resembling the two-metal-assisted mechanism suggested for structurally related adenylate cyclases and polymerases (Chan et al. 2004; Wassmann et al. 2007). Most active DGCs are subject to allosteric product inhibition. A conserved RXXD motif (inhibitory site, I-site; with X standing for any amino acid) in close proximity to the A-site participates in binding of a c-di-GMP dimer, which inhibits DGC activity (Chan et al. 2004; Christen et al. 2006). Feedback control of DGC activity ensures prevention of excessive GTP consumption, establishes an upper limit for the cellular c-di-GMP concentration and increases stability of c-di-GMP signalling towards stochastic perturbations (Christen et al. 2006).

Degradation of c-di-GMP is mediated by the EAL domain of c-di-GMP-specific phosphodiesterases (PDEs), which specifically cleaves c-di-GMP into its linear form 5´pGpG (assigned as PDE-A activity) that is then further degraded by an as yet uncharacterized, comparably slow and most likely unspecific reaction to GMP (termed PDE-B activity) (Christen et al. 2005; Schmidt et al. 2005). The residues required for activity exceed those

found in the EAL site and hydrolysis of c-di-GMP is thought to be mediated by a general base-catalyzed mechanism involving Mg^{2+} (or Mn^{2+}) (Rao et al. 2008).

Another less frequent domain involved in the turnover of c-di-GMP is the HD-GYP domain, which constitutes a subgroup of the HD superfamily of metal-dependent phosphohydrolases (Galperin et al. 1999). PDE activity of the HD-GYP domain converts c-di-GMP to GMP via pGpG, indicating that the HD-GYP domain might differ from the EAL domain in its activity against pGpG (Ryan et al. 2006).

GGDEF domains are often found together with EAL or HD-GYP domains in a single polypeptide. The coexistence of opposing enzymatic activities in a single protein has long been controversial and only a few reports suggest that bifunctional proteins may exist (Tarutina et al. 2006; Ferreira et al. 2008). However, recent studies on two homologous GGDEF and EAL domain containing proteins from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* unequivocally attributed both diguanylate cyclase (DGC) and phosphodiesterase (PDE) activity to the full length proteins *in vitro* (Kumar and Chatterji 2008; Gupta et al. 2010). Whether both activities are relevant *in vivo* and how they are coordinated to prevent a futile cycle of c-di-GMP synthesis and degradation remains to be shown. However, the majority of composite proteins studied *in vitro* so far, only shows either PDE or DGC activity (Christen et al. 2005; Schmidt et al. 2005; Takahashi and Shimizu 2006). Often, one domain shows deviations from the conserved residues required for activity thus accounting for the enzymatic inactivity of this domain. In a GGDEF-EAL protein from *Caulobacter crescentus* a catalytically inactive GGDEF domain plays a role in allosteric regulation of the neighbouring EAL domain by GTP. The GEDEF sequence in the active site of the enzyme is still able to bind GTP and in turn exerts a stimulatory effect on the PDE activity of the EAL domain (Christen et al. 2005).

Finally, in addition to GGDEF and EAL domains involved in the "making and breaking" of c-di-GMP and to the recruitment of catalytically inactive GGDEF domains as sensory domains in composite proteins, an increasing number of reports indicate that some proteins with GGDEF and EAL domains do not show any c-di-GMP-associated activity at all, but instead have adopted c-di-GMP-unrelated functions. In these cases the components of the c-di-GMP signalling system have lost their original activities and often show highly degenerate GGDEF and EAL motifs (Suzuki et al. 2006; Tschowri et al. 2008). These "non-c-di-GMP-associated" GGDEF and EAL proteins have been shown to control such diverse processes as degradation of non-coding RNAs (Suzuki et al. 2006), biofilm gene expression (Holland et al. 2008), motility (Simm et al. 2009; Wada et al. 2011) and transcriptional

regulation in response to blue-light (Tschowri et al. 2008). The latter case involves a BLUF-EAL domain protein, which acts as an anti-repressor that directly interacts with a repressor and releases it from its operator in response to blue light irradiation (Tschowri et al. 2008).

The majority of GGDEF, EAL and HD-GYP domains is joined to N-terminal sensory input domains comprising transmembrane sensor domains as well as phosphoacceptor and other cytoplasmic sensor domains (Galperin et al. 2001; Galperin 2004). The great diversity of these sensory input domains suggests that the c-di-GMP signalling system is able to integrate a large variety of signals. Signal transduction in response to various parameters such as oxygen or redox conditions, blue light and cell cycle signals has been reported for different GGDEF and EAL proteins (Jenal and Malone 2006; Tamayo et al. 2007).

C-di-GMP signalling displays a high diversity concerning the effector components that bind the second messenger and mediate downstream effects. The PilZ protein domain, named after its presence in a *Pseudomonas aeruginosa* protein involved in twitching motility, was the first of the long-sought c-di-GMP effectors to be discovered (Amikam and Galperin 2006). c-di-GMP binding to a conserved RXXXR motif (with X standing for any amino acid) at the N-terminal end of the PilZ domain is an essential step in c-di-GMP-mediated regulation of motility, extracellular polysaccharide synthesis, biofilm formation and virulence in many organisms (Ryjenkov et al. 2006; Christen et al. 2007; Merighi et al. 2007; Pratt et al. 2007).

Following the discovery of the PilZ protein domain, c-di-GMP binding to other effector proteins was shown. In *P. aeruginosa* the transcription factor FleQ as well as a putative inner membrane protein, PelD, both involved in the expression of extracellular polysaccharides, specifically bind c-di-GMP, the latter protein employing a c-di-GMP binding site very similar to the I-site shown to be involved in feedback inhibition of DGCs (see above) (Lee et al. 2007; Hickman and Harwood 2008). Another transcription factor, the CRP-like protein Clp from *Xanthomonas* is allosterically inhibited in its DNA binding activity necessary for virulence gene regulation upon c-di-GMP binding (Leduc and Roberts 2009; Chin et al. 2010; Tao et al. 2010). Interestingly, the GGDEF protein PopA from *Caulobacter crescentus*, which is involved in cell cycle regulation, cannot synthesize c-di-GMP due to a degenerate GGDEF motif, but retained its ability to bind c-di-GMP via a conserved I-site, thereby adopting its function as an effector (Duerig et al. 2009). Similarly, CdgG from *Vibrio cholerae*, a GGDEF protein participating in the regulation of rugosity, biofilm formation and motility requires an intact I-site motif, but not its degenerate active site for regulation, suggesting it as another I-site-dependent c-di-GMP binding effector (Beyhan et al. 2008). Degenerate EAL domains can

also be employed for c-di-GMP binding by effector proteins as observed with the inner membrane protein LapD from *Pseudomonas fluorescens* (Newell et al. 2009) and the GGDEF-EAL domain protein FimX from *Pseudomonas aeruginosa* (Navarro et al. 2009; Qi et al. 2010).

Inverse c-di-GMP-mediated control of extracellular matrix components and motility in *V. cholera* is mediated by the response regulator VspT. DNA-binding and transcriptional regulation by VspT were recently shown to be regulated by c-di-GMP binding to its receiver domain, which induces oligomerization of VpsT (Krasteva et al. 2010). This represents an interesting deviation from the conventional mechanism of phosphorylation-dependent regulation of response regulator activity.

Finally, two different riboswitches have recently been discovered as direct c-di-GMP-binding targets. The first riboswitch, termed GEMM (Genes for the Environment, for Membranes and Motility) RNAs after the physiological context of the genes possibly regulated by this motif, is found in the untranslated regions of different mRNAs in several bacterial species and responds to c-di-GMP binding with structural changes that alter the expression of downstream genes (Sudarsan et al. 2008). A different c-di-GMP binding riboswitch is used by *Clostridium difficile* to mediate alternative RNA processing within the 5`-untranslated region of a putative virulence gene (Lee et al. 2010). Incomplete splicing in the absence of c-di-GMP, yields a mRNA that cannot be translated, while splicing in the presence of c-di-GMP results in the production of a translation-proficient mRNA species (Lee et al. 2010). These RNA-based targets emphasize the functional flexibility and versatility provided by the nucleotide second messenger c-di-GMP, which, in its molecular nature, represents a RNA species.

Reflecting the diversity of effector components, the molecular targets of c-di-GMP are found at many different regulatory levels. In addition to allosteric regulation of enzyme function (Ross et al. 1987; Weinhouse et al. 1997; Amikam and Galperin 2006) and interference with the assembly and function of more complex cellular structures such as pili (Huang et al. 2003; Kazmierczak et al. 2006) or the flagellar motor (Christen et al. 2007), c-di-GMP also targets gene transcription (Weber et al. 2006; Hickman and Harwood 2008; Sudarsan et al. 2008) and, as recently shown in *Caulobacter crescentus*, c-di-GMP also participates in the regulation of localized proteolysis (Duerig et al. 2009).

1.2.2.2 Signalling specificity and the possibility of local signalling

Comparative analysis of complete microbial genomes revealed the presence of multiple copies of genes encoding GGDEF, EAL and HD-GYP domains in the genomes of most bacterial species, while they are absent from the sequenced genomes of the Archaea and Eukaryotes (Galperin et al. 2001; Galperin 2004). The numbers of GGDEF, EAL and HD-GYP encoding genes vary vastly between the species, with generally higher numbers in gram-negative bacteria (e.g. *Escherichia coli* encoding 19 GGDEF and 17 EAL domains) than in gram-positive bacteria (e.g. *Bacillus subtilis* encoding 4 GGDEF and 3 EAL domains) and their high abundance in free-living bacteria mirrors their importance in signal transduction and adaptational processes in complex environments (Galperin et al. 2001). Considering this abundance of proteins with redundant enzymatic activities within one organism, one of the most puzzling aspects of c-di-GMP-mediated signalling remains the question of how signalling specificity can be achieved in these systems. Although the molecular details of how single GGDEF, EAL and HD-GYP domain proteins within such complex networks are able to specifically affect certain c-di-GMP-dependent target processes remain mostly elusive, sequestration of c-di-GMP control systems has been suggested as a solution to this problem (Jenal and Malone 2006; Kader et al. 2006; Ryan et al. 2006; Hengge 2009a; Christen et al. 2010; Hengge 2010b; Ryan et al. 2010). Different modes of sequestration can be envisaged: Through temporal regulatory sequestration only a subgroup of the DGCs and PDEs encoded in the genome could be present and active at any given point in the cell cycle or under any growth condition. This could be realized through specific regulatory expression patterns of the corresponding genes combined with tight control of enzymatic activities through the N-terminal sensory input domains. Alternatively, certain DGCs and PDEs might form complexes with their respective effector and target molecules yielding microcompartments in which the components of one c-di-GMP signalling module are functionally sequestered. Similarly, spatial restriction of components of the c-di-GMP signalling network to specific locations within the cell, for example to the cell poles, could confine specific c-di-GMP signalling to the immediate vicinity of co-localized DGCs, PDEs, effector and target molecules.

1.2.2.3 Physiological functions regulated by c-di-GMP

Rapid research over the last few years led to the identification of a variety of processes which are subject to c-di-GMP-mediated regulation. As a consequence, a general role of c-di-GMP

in promoting functions associated with bacterial biofilm formation and sessility and as a negative regulator of motility and virulence arose.

In *Escherichia coli* and *Salmonella*, regulation of the major biofilm components adhesive curli fimbriae and cellulose is under c-di-GMP control. Elucidating details of c-di-GMP-mediated regulation of curli expression was one subject of this work and the architecture of the control cascade directing curli synthesis will be described below (see 1.3.2).

C-di-GMP control of adhesive structure expression also plays a role in the transition from motile swarmer to sessile stalked cells that is part of the cell cycle of *Caulobacter crescentus*. The response regulator and DGC PleD plays an essential role in remodelling of the pole during this transition, which involves loss of pili and flagellum and synthesis of a stalk with an adhesive holdfast. PleD is evenly distributed in the swarmer cell, but concentrates at the emerging stalked pole during swarmer-to-stalked cell transition (Paul et al. 2004). Targeting to this pole is mediated by phosphorylation of PleD, which induces dimerization, a prerequisite for both DGC activity of the PleD GGDEF domain and polar localization of the protein (Paul et al. 2004; Paul et al. 2007). PleD activity is required for the essential steps in swarmer-to-stalked cell transition (Hecht and Newton 1995; Aldridge and Jenal 1999; Aldridge et al. 2003). Simultaneous sequestration and activation of DGC activity strongly indicates local production of c-di-GMP at the incipient stalked pole, resulting in inhibition of motility and induction of adhesive structures.

In addition, c-di-GMP also contributes to the control of the cell cycle itself in *Caulobacter crescentus*. Upon c-di-GMP binding to its I-site, the degenerate GGDEF protein PopA is sequestered to the incipient stalked pole during swarmer-to-stalked cell transition. Via the mediator protein RcdA, PopA targets the cell cycle regulator CtrA to this pole, where it is degraded by the co-localized ClpXP protease, thus relieving CtrA-mediated inhibition of replication and cell cycle progression (Duerig et al. 2009).

The causative agent of the severe diarrheal disease cholera, *Vibrio cholerae*, represents a paradigm organism for the role of c-di-GMP signalling in virulence gene regulation. In the classical biotype, c-di-GMP degradation by the EAL domain protein VieA is required for virulence gene expression (Tischler and Camilli 2005), reflecting the requirement for low c-di-GMP levels for the synthesis of virulence factors that has been observed in many pathogenic bacteria. On the other hand, VieA-mediated c-di-GMP degradation negatively influences biofilm formation (Tischler and Camilli 2004). This inverse regulation is thought

to play a role in the transition of *V. cholerae* between the host, where virulence factor expression is pivotal and the aquatic environment, where biofilms are formed.

Although the function of c-di-GMP varies vastly in these different species with their different physiological and ecological niches, a common role for c-di-GMP as a regulator of bacterial lifestyle transitions can be inferred, e.g. in the transition between motile and sedentary cells, as described above for *C. crescentus* or in the transition between the host and the environment in pathogenic bacteria such as *V. cholerae*.

1.3 Regulation of motility and adhesion in *Escherichia coli*: Integrated regulation by complex transcriptional networks and c-di-GMP control

1.3.1 Bacterial biofilm formation

In their natural environments bacteria often grow in communities that are associated with surfaces and embedded in an extracellular matrix (Stoodley et al. 2002; Vlamakis and Kolter 2010). These single- or mixed-species microbial communities, termed biofilms, provide cells with specific benefits that are the essence of the adverse impact of microbial biofilm formation in medical and industrial settings. Enclosure in an extracellular matrix protects bacteria from detrimental influences and physical forces such as antimicrobial chemicals, attacks from the host immune system, shearing forces and desiccation. Growing on industrial devices, biofilms can thus cause severe problems such as clogging or corrosion, e.g. of pipes. In medical settings the impact of biofilm formation is manifold. Growth on medical devices can lead to chronic infections that are difficult to eradicate, and chronic infections associated with biofilm formation also cause serious problems e.g. in cystic fibrosis patients and in other diseases (Donlan and Costerton 2002). In addition, biofilm formation seems to play a role in the persistence and transmission of pathogenic bacteria in natural environments (Huq et al. 2008). Induced biofilm formation in response to subinhibitory concentrations of antibiotics, as shown in several pathogenic bacteria ((Boehm et al. 2009) and references cited therein), further adds to the complex role biofilm formation plays in pathogenicity.

The lifestyle change that bacteria undergo when changing from the planktonic, single-cell and motile state to the adhesive, multicellular biofilm lifestyle, is a multi-step process (Fig. 1.5) (Stoodley et al. 2002; Beloin et al. 2008; Monds and O'Toole 2009). Following

initial and *reversible attachment* of planktonic cells to a surface, bacteria start to secrete exopolysaccharides and surface contacts are strengthened with the help of adhesins leading to *irreversible adhesion* and the formation of *microcolonies*. These microcolonies *mature* into complex structures termed *macrocolonies*, from which individual cells or cell groups may eventually detach and revert to the planktonic lifestyle.

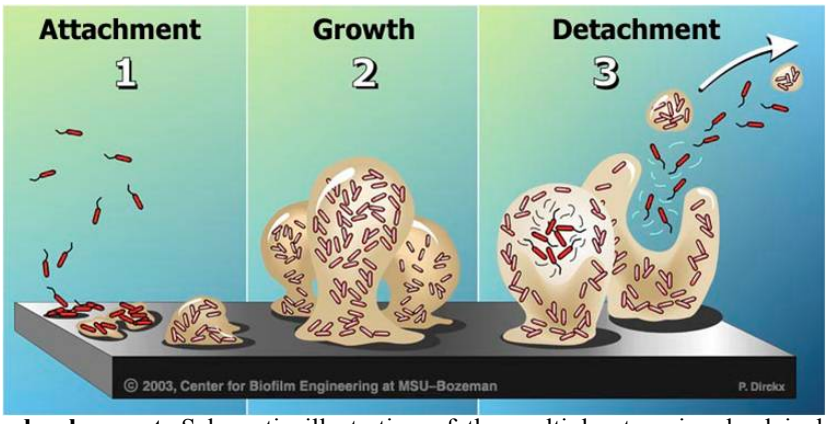

Fig. 1.5: Biofilm development. Schematic illustration of the multiple steps involved in biofilm formation. Initial, reversible attachment of planktonic cells is followed by irreversible attachment and formation of microcolonies that grow and mature into more complex macrocolonies from which cells may eventually detach. (This figure was obtained from the website of the Montana State University Centre for Biofilm Engineering (http://www2.erc.montana.edu/)).

Research on bacterial biofilms has uncovered many common principles associated with biofilm formation in different species, e.g. with respect to the benefits provided to cells in a biofilm or concerning regulatory principles such as induction of biofilm formation by high c-di-GMP levels (see 1.2.2). However, biofilm formation differs significantly amongst species and even amongst different strains of the same species (Vlamakis and Kolter 2010). In addition, biofilm formation of a species varies depending on environmental conditions. Major differences can be found in the structural features of bacterial biofilms, i.e. in the composition of the extracellular matrix and the presence of structural elements such as adhesins. The extracellular matrix that has an important structural and protective role in the biofilm mainly consists of water, polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender 2010). A vast variety of polysaccharides has been found in the extracellular matrices of bacterial biofilms including e.g. cellulose, poly-ß-1,6-N-acetyl-glucosamine (PGA) and colanic acid, compounds that can be observed in certain *E. coli* biofilms depending on the strain and growth conditions (Beloin et al. 2008). Analogously, various different protein adhesins have been observed to contribute to attachment of cells within a biofilm in different settings. In *E. coli* these include e.g. curli and type 1 fimbriae, Antigen 43 and conjugative pili (Beloin et al. 2008). Flagella-mediated motility seems to play a role in

biofilm formation and in particular during initial adhesion, mainly by allowing cells to reach and spread over a surface (Pratt and Kolter 1998; Wood et al. 2006). However, the contribution of motility to biofilm formation is not essential in all biofilms and also seems to depend on the strain and the conditions under which the biofilm is formed (Pratt and Kolter 1999; Prigent-Combaret et al. 2000; Sheikh et al. 2001).

Variation exists also with respect to underlying regulatory mechanisms directing biofilm formation, with differences often reflecting the ecological niche occupied by the respective organism. For example, in *V. cholera* several regulatory connections between the quorum sensing system and c-di-GMP signalling contribute to the coordination of biofilm formation (Kovacikova et al. 2005; Lim et al. 2007; Hammer and Bassler 2008; Waters et al. 2008). This link between c-di-GMP signalling and quorum sensing results in an integration of information about both the surrounding bacterial population and the local environment into biofilm formation. Population density is likely to be an essential factor influencing the behaviour of bacteria both in the host and in a biofilm-inducing environment, thus providing a rationale for the connection of the two systems in this pathogen.

These variations in biofilm formation within and between single species indicate the need for a careful choice of model systems for biofilm formation. A well established model for biofilm formation in *Salmonella* is the *rdar* (red, dry and rough) colony morphotype, a biofilm formed at the wet surface/air interface by cells grown on agar plates supplemented with the dye congo red (Römling 2005). The *rdar* morphotype is characterized by the production of curli fimbriae and cellulose (see 1.3.2), two extracellular matrix components that are also produced by *E. coli* (Zogaj et al. 2001; Römling 2005; Weber et al. 2006). Moreover, *E. coli* represents a convenient model system for studying the transition from planktonic growth to the sedentary state, as this lifestyle-switch can be observed during the transition from post-exponential growth to stationary phase with cells grown in complex medium. This growth phase dependence of the lifestyle manifests itself in a peak of flagellar motility in post-exponential phase, when cells are foraging for nutrients, followed by a reduction in motility and concomitant induction of adhesive structures such as curli fimbriae when cells enter stationary phase (Adler and Templeton 1967; Amsler et al. 1993; Arnqvist et al. 1994; Weber et al. 2006; Zhao et al. 2007).

When this thesis was started, the molecular mechanisms controlling this growth phase-dependent lifestyle-switch were unknown. As the elucidation of these regulatory mechanisms was a central focus of this work, the last two sections of this introduction will give a more detailed description of the two main features characterizing the two lifestyles, i.e. curli

fimbriae expression and flagella-driven motility. Figure 1.6 shows the basic architecture of the cascades regulating motility during post-exponential growth and curli expression in stationary phase, while details on both cascades will be given below.

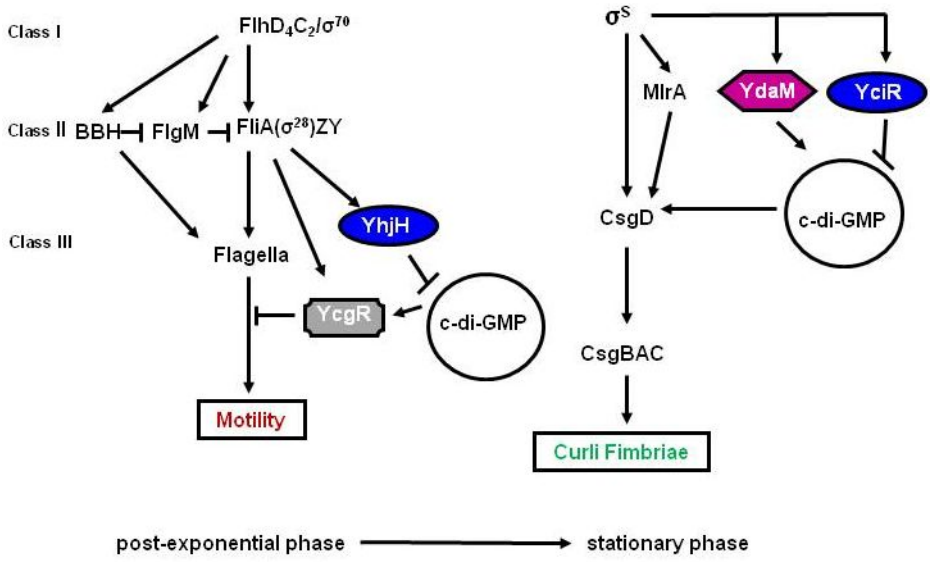

Fig. 1.6: Basic architecture of the cascades controlling $E\sigma^{70}/\sigma^{28}$-directed flagellar motility in post-exponential phase and $E\sigma^{S}$-induced curli expression in stationary phase. A simplified view of the hierarchical structure of the flagellar gene regulon is given on the left, with the class I master regulator $FlhD_4C_2$ and selected class II and III gene products shown. BBH indicates the flagellar basal body and hook, specified by class II genes, while class III genes encode structural and regulatory components that are required to build functional flagella, and components of a c-di-GMP control module. The most important regulators involved in expression of curli expression and their hierarchical expression are shown on the right side. C-di-GMP-specific phosphodiesterases are coloured blue, diguanylate cyclases pink. For details refer to the following sections.

1.3.2 Regulation of curli fimbriae formation in *E. coli*

Curli fimbriae are amyloid fimbriae produced by *E. coli* and *Salmonella* as part of the extracellular matrix of the rdar morphotype (see above). By mediating contacts to both abiotic and biotic surfaces, curli fimbriae promote cell-cell as well as cell-surface contacts and thereby seem to play a predominant role in the initial stages of biofilm formation, but also in attachment to and invasion of host cells in pathogenic bacteria and in adherence to plant tissue (Prigent-Combaret et al. 2001; Barnhart and Chapman 2006).

The genes required for curli fimbriae expression are organized in two divergently transcribed operons, separated by an intergenic region of approximately 750 nucleotides that contains the two promoters. The *csgBAC* operon encodes the structural curli components, i.e. the major curli subunit CsgA, the curli nucleator protein CsgB, which primes self-assembly of CsgA on the cell surface (Hammar et al. 1995; Hammar et al. 1996), and the uncharacterized CsgC. The *csgDEFG* operon specifies CsgD, a transcriptional regulator of the NarL/FixJ

family of response regulators with a LuxR-like helix-turn helix DNA-binding motif, and CsgE, CsgF and CsgG, accessory proteins that participate in the secretion and assembly process of curli fimbriae (Chapman et al. 2002; Robinson et al. 2006; Epstein et al. 2009; Nenninger et al. 2009).

Regulation of curli fimbriae expression is complex. As mentioned above, curli expression is induced when cells enter the stationary phase of growth. This reflects the fact that curli expression is part of the σ^S-mediated general stress response, both in *E. coli* and *Salmonella* (Olsen et al. 1989; Arnqvist et al. 1992; Arnqvist et al. 1994). σ^S controls curli expression in these organisms by multiple feedforward control of a complex transcription factor cascade (Fig. 1.6). σ^S regulates the expression of the MerR-like regulator MlrA, which, together with σ^S, activates the transcription of the essential curli regulator CsgD, and CsgD in turn induces the expression of the *csgBAC* operon (Olsen et al. 1993; Arnqvist et al. 1994; Hammar et al. 1995; Römling et al. 1998; Brown et al. 2001; Zakikhany et al. 2010). Directly preceding the beginning of this work, the Hengge group demonstrated that in *E. coli* σ^S also controls the expression of the GGDEF domain protein and DGC YdaM and the composite GGDEF-EAL protein and PDE YciR. These two proteins antagonistically control curli expression at the level of *csgD* transcription (Weber et al. 2006). C-di-GMP synthesized by YdaM is absolutely required for *csgD* transcription, while YciR counteracts YdaM activity.

CsgD also activates cellulose synthesis by inducing the expression of YaiC (or the *Salmonella* homologue AdrA), a GGDEF protein whose DGC activity is required for cellulose synthase activity (Römling et al. 2000; Zogaj et al. 2001; Simm et al. 2004). However, like many laboratory strains, the *E. coli* strains used in this study do not produce cellulose. Amongst other CsgD targets observed in various strain backgrounds is also the *iraP* gene, coding for an anti-adaptor protein that inhibits RssB-mediated degradation of σ^S (Bougdour et al. 2006; Brombacher et al. 2006). Induction of *iraP* was shown to be responsible for a CsgD-mediated increase in σ^S stability and σ^S-dependent gene expression, thus establishing positive feedback regulation that coordinates biofilm formation with the σ^S-mediated general stress response (Gualdi et al. 2007).

In addition to being part of the general stress response (and thereby also being subject to indirect regulation by factors that regulate σ^S levels and activity, e.g. stresses inducing σ^S expression, Crl, (p)ppGpp, sigma factor competition), curli fimbriae expression is directly regulated in response to a multitude of environmental stimuli. In most *E. coli* species, curli fimbriae expression is restricted to temperatures below 30° (Olsen et al. 1989; Arnqvist et al. 1992), however, curli expression at 37°C has been observed with some commensal isolates

and pathogenic strains and under specific conditions (Bokranz et al. 2005; Kikuchi et al. 2005; Szabo et al. 2005). Examples of other environmental stimuli affecting curli fimbriae expression include oxygen availability (Bokranz et al. 2005) and osmolarity (Prigent-Combaret et al. 2001).

Signal transduction in response to the majority of environmental stimuli is integrated into the curli control cascade at the level of *csgD* transcription. Analogous to the situation in *Salmonella* (Gerstel et al. 2003; Gerstel and Römling 2003) the *csgD* promoter region in *E. coli* is bound by a large number of regulatory proteins, including both nucleoid-associated proteins and response regulators of two-component signal transduction systems (Ogasawara et al. 2010a). The nucleoid-associated proteins H-NS and IHF bind to multiple sites, exerting negative and positive influences on *csgD* expression, respectively (Ogasawara et al. 2010a). However, an additional and dominating positive role of H-NS in *csgD* transcription has been described in *Salmonella* (Gerstel et al. 2003), but the mechanism underlying this observation was unknown at the beginning of this work. Several two-component systems are involved in control of *csgD* expression. Two-component systems are signal transduction systems that, in their simplest form, comprise a histidine kinase that autophosphorylates in response to an environmental stimulus and a response regulator with a receiver domain to which the phosphate is subsequently transferred, thereby activating a specific output function (e.g. DNA binding or an enzymatic activity) exerted by another domain of the protein (Stock et al. 2000). The response regulators CpxR, OmpR and RstA have been shown to directly bind to the *csgD* promoter region, stimulating (OmpR, RstA, the latter exerting its positive control only at low pH) or repressing (CpxR) *csgD* transcription (Prigent-Combaret et al. 2001; Ogasawara et al. 2007; Ogasawara et al. 2010a). The CpxRA two-component system has been implied in sensing of surfaces (Otto and Silhavy 2002). Similarly, the EnvZ/OmpR system, which is known to respond to changes in osmolarity, might sense surfaces through local modifications in osmolarity that are often found on surfaces due to absorbtion of organic molecules (Beloin et al. 2008). This underscores the important role of these two-component systems in coordinating biofilm formation. The RcsCDB two component system, which, amongst others, regulates capsular polysaccharide synthesis, has also been implicated in surface sensing and regulation of curli fimbriae expression (Ferrieres and Clarke 2003; Majdalani and Gottesman 2005; Vianney et al. 2005; Ferrieres et al. 2009).

Binding of such a large number of regulators within the *csgD* promoter region (in some cases to partially overlapping sites) suggests a complex interplay between the involved proteins and two-component systems in regulation of *csgD* transcription (Jubelin et al. 2005;

Ogasawara et al. 2010a). Despite its well established role in *csgD* transcriptional induction, the exact binding site(s) of the transcriptional regulator MlrA and its role in this nucleoprotein complex assembled at the *csgD* promoter were not known at the beginning of this thesis.

Additional complexity is added to curli control by the recent discovery of several small regulatory RNAs involved in the post-transcriptional control of *csgD* expression (Holmqvist et al. 2010; Mika et al. 2011). Moreover, the degenerate GGDEF/EAL protein, YhdA, is involved in curli fimbriae synthesis by affecting the expression of both *csgD* and the essential curli activator gene *ydaM* (Sommerfeldt et al. 2009). YhdA was shown to affect the turnover of small regulatory RNAs in the Csr system (Suzuki et al. 2006), a system involved in post-transcriptional control of many genes through the RNA-binding regulator CsrA (Timmermans and Van Melderen 2010), thus suggesting another small RNA-based mechanism in curli fimbriae control.

Interestingly, CsgD belongs to the family of response regulators. Although a conserved aspartate residue that is the site of phosphorylation in the receiver domain of many response regulators is also present in CsgD, other conserved residues known to be required for phosphorylation are missing. Nevertheless, a recent report suggests that in *Salmonella* CsgD can be phosphorylated and that phosphorylation negatively affects CsgD binding to target promoters and consequently its function in promoting biofilm formation (Zakikhany et al. 2010).

Taken together, strong σ^S dependence of curli fimbriae expression and the resulting induction by stimuli that induce the σ^S regulon, in combination with theses direct regulatory influences establish tight regulation of curli fimbriae expression in response to environmental signals. Many of these signals feed into the curli control cascade firstly by affecting σ^S induction and, in addition, through regulatory systems affecting *csgD* expression. Despite the large number of known curli regulators, it was clear at the beginning of this work that our knowledge of regulatory mechanisms governing curli fimbriae expression was far from being complete. Open questions concerned e.g. details on the input of c-di-GMP into curli fimbriae regulation and the coordination of curli fimbriae expression with flagellar motility. Elucidation of some of these mechanisms was the major aim of this thesis.

1.3.3 Regulation of motility in *E. coli*

Flagella are complex macromolecular machines that enable bacteria to move along gradients and thus towards favourable and away from unfavourable environments. The arrangement of flagella on the bacterial cell varies between bacterial species. *E. coli* and *Salmonella* show peritrichous (also called lateral) flagellation, i.e. multiple flagella spread over the cell surface, while e.g. *Vibro cholerae* harbours only a single polar flagellum. Structure and function of the flagellum, as well as the architecture of the genetic network directing its expression are highly conserved amongst Gram-negative enteric bacteria such as *E. coli* and *Salmonella*.

1.3.3.1 Structure and function of the flagellum

Fig. 1.7A shows the structure and subunit composition of the *Salmonella enterica* serovar Typhimurium flagellum, which is conserved in *E. coli*. The flagellum can be roughly divided into three parts, the basal body that is inserted into the cell envelope, the extracellular filament and the hook that connects them (Chevance and Hughes 2008). The basal body comprises the engine of the flagellum. It consists of a proton-conducting stator, formed by the cytoplasmic membrane proteins MotA and MotB, that energizes flagellar rotation and of the rotor, a ring structure (also called C ring or switch complex) composed of the FliG, FliM and FliN proteins, that interacts with the stator complex. This interaction, mainly between MotA and FliG, causes rotation, which can be either anticlockwise, resulting in formation of a bundle of several flagella that enables cells to swim smoothly, or clockwise, causing bacteria to tumble and reorient due to bundle disintegration (Blair 2003). The rotor is attached to the MS ring, a ring structure that acts as an inner membrane anchor. The MS ring is associated with type III secretion system components on its cytoplasmic side and with a hollow rod that extends from the rotor through the periplasmic space. Two ring structures, the P and L rings, function as rod bushings in the peptidoglycan layer and outer membrane, respectively. The hook, which is built at the cell surface, flexibly connects the filament to the basal body via a hook-filament junction. The hollow filament is built by polymerization of FliC proteins beneath the filament cap, formed by FliD. The hook-basal body complex of the flagellum represents a type III secretion system with similarity to type III secretion systems used by pathogenic bacteria (Blocker et al. 2003) and most flagellar components are secreted by this mechanism, which is driven by proton influx and uses secretion chaperones and an ATPase complex to stabilize and deliver secretion substrates to the secretion machinery (Chevance and Hughes 2008).

An elaborate chemotaxis system regulates switching between counterclockwise and clockwise motor rotation and thereby between swimming and tumbling in response to attractant and repellent gradients (Fig. 1.7B) (Wadhams and Armitage 2004). The sequence of signalling events is well understood and will be summarized here using the example of chemotaxis along attractant gradients. Transmembrane receptors, called methyl-accepting chemotaxis proteins, *stimulate* autophosphorylation of the histidine kinase CheA in response to a *decrease* in attractant binding and *repress* autophosphorylation upon *increased* attractant binding. Autophosphorylated CheA transfers the phosphate to the response regulator CheY, which in turn interacts with the flagellar rotor protein FliM, causing the rotor to switch to clockwise rotation, thus promoting tumbling. Consequently, low levels of phosphorylated CheY in response to increased attractant binding reduce clockwise rotor switching and result in prolonged run-phases up the attractant gradient.

Temporal comparison of attractant concentrations is achieved through receptor adaptation (Vladimirov and Sourjik 2009). The constitutively active methyltransferase CheR methylates glutamate residues within the chemoreceptor proteins, whereas the response regulator and methylesterase CheB decreases receptor methylation. Methylation induces activity of the receptor, resulting in enhanced autophosphorylation of CheA, even in the presence of attractants. CheB is activated by phosphorylation through CheA. Thus, increased attractant concentrations initially repress CheA autophosphorylation, but at the same time CheB is not activated and does not interfere with receptor methylation by CheR, which results in increased CheA activation and eventually resets the system to the state before stimulation. After this adaptation, any subsequent changes in attractant concentration can be sensed. Conversely, a decrease in attractant binding initially results in CheA autophosphorylation, followed by CheB-mediated reduction of receptor methylation and the concomitant decrease in CheA activation again re-establishes the pre-stimulus state (Wadhams and Armitage 2004; Vladimirov and Sourjik 2009).

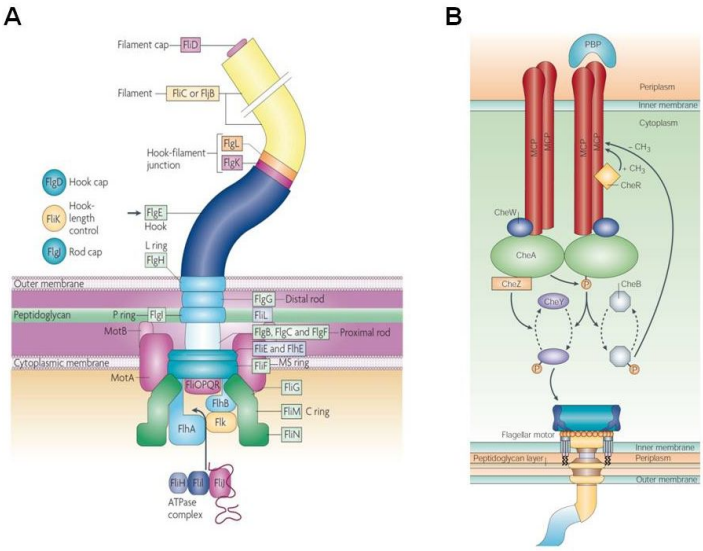

Fig. 1.7: Structure and function of the bacterial flagellum. (A) Structure and subunit composition of the *Salmonella enteric* serovar Typhimurium flagellum. The proteins building the basal-body structures described in the text, the hook and the filament are indicated. FliO, FliP, FliQ and FliR (indicated as FliOPQR), as well as FlhA, FlhB and Flk are components of the type III secretion system. FliH; FliI and FliJ are components of the cytoplasmic ATPase complex that helps to deliver secretion sub-strates to increase efficiency of the assembly process. The rod is divided into proximal and distal parts that differ in subunit composition. FljB is an alternative filament subunit in *Salmonella* species. FlgD and FlgJ denote hook and rod cap proteins, respectively, that cap the growing hook and assist in assembly. FliK is a protein involved in mediating the change in flagellar secretion specificity from rod and hook substrates to filament substrates once the hook has reached a certain length. (Figure A is a reproduction from (Chevance and Hughes 2008)) **(B)** Illustration of the chemotactic system in *Escherichia coli*. Components involved in chemotactic responses and the regulatory interactions described in the text are shown. MCP denotes the methyl-accepting chemotaxis proteins that in addition to directly binding ligands can also indirectly bind them through a periplasmic binding protein (PBP). CheW is an adaptor protein linking the methyl-accepting chemotaxis proteins to CheA. The phosphatase CheZ increases turnover of phosphorylated CheY, thereby terminating the signal. (Figure B is a reproduction from (Wadhams and Armitage 2004)).

1.3.3.2 The flagellar gene regulon

Flagellar motility is a very energy-consuming process, with respect to both synthesis and function of the flagellum. Accordingly, expression of flagella is tightly regulated not only in response to environmental signals, but also in response to signals that monitor progression of flagellar assembly. In enteric bacteria such as *E. coli* and *Salmonella*, this is realized by the hierarchical expression of three classes of flagellar genes in a temporal order and involves sophisticated mechanisms coupling progression in the gene expression cascade with completion of morphological structures (Fig. 1.6) (Chilcott and Hughes 2000; Aldridge and Hughes 2002; Soutourina and Bertin 2003). The first class of flagellar genes comprises the single σ^{70}-dependent *flhDC* operon, encoding the components of the master regulator complex $FlhD_4C_2$. $FlhD_4C_2$ in conjunction with σ^{70} induces transcription of the class II genes, most of which specify components of the hook-basal body complex. In addition, the *fliAZY* operon belongs to this class. *fliA* encodes the alternative sigma factor σ^{28} that in turn activates

transcription of class III genes. Class III flagellar genes code for the subunits of the flagellar filament, the stator subunits and a number of regulatory proteins, including chemotaxis proteins. In addition, the class III genes *yhjH*, encoding an EAL protein and *ycgR*, encoding a c-di-GMP-binding PilZ protein were shown to have interdependent positive and negative effects, respectively, on motility (Ko and Park 2000b; Rychlik et al. 2002; Frye et al. 2006; Ryjenkov et al. 2006). However, not all details of c-di-GMP-meditated regulation of motility involving these class III gene products had been clarified at the beginning of this thesis.

The different classes of genes are distinguished by differences in their promoter sequences that establish expression control by $E\sigma^{70}/FlhD_4C_2$ (class II) or $E\sigma^{28}$ (class III). However, several flagellar genes, including the *fliAZY* operon are under the control of both class II and class III promoters (Mytelka and Chamberlin 1996).

Coupling of gene expression with assembly is realized with the help of the anti-σ^{28} factor FlgM. FlgM is also expressed from both class II and III promoters (Kutsukake 1994). During class II gene expression, FlgM binds to σ^{28} and inhibits class III gene expression (Ohnishi et al. 1992; Chadsey et al. 1998). Since FlgM represents a secretion substrate of the flagellar type III secretion system, it is exported from the cell upon completion of hook-basal body complex assembly, thus releasing σ^{28} that can then commence transcription of class III genes (Hughes et al. 1993; Kutsukake 1994; Karlinsey et al. 2000). The regulatory interaction between σ^{28} and FlgM is multifaceted. FlgM does not only inhibit σ^{28} transcriptional activity, but also protects the sigma factor from proteolysis, which is mainly mediated by Lon protease (Barembruch and Hengge 2007). Proteolysis of σ^{28} in the absence of FlgM thus correlates with induction of class III gene expression. The function of this regulatory interaction seems to lie in the proper coordination of class III gene expression with class II gene expression, which is essential for coordinate synthesis and proper function of the flagellum. σ^{28} proteolysis rapidly re-establishes a stoichiometric balance between σ^{28} and its anti-sigma factor upon secretion of the latter. This avoids over-activation of class III gene expression and also essentially contributes to couple class II and III gene expression, i.e. to shut down class III gene expression upon down-regulation of class II gene expression (Barembruch and Hengge 2007). Proteolysis also plays a role in the control of the master regulator $FlhD_4C_2$, which is subject to ClpXP-mediated degradation (Tomoyasu et al. 2003), the physiological function of which, however, has remained undetermined.

In addition to the above mentioned functions, the interaction between σ^{28} and FlgM is also involved in mediating FlgM secretion. Interestingly, σ^{28} was found to function as the secretion chaperone for FlgM, facilitating FlgM export through the flagellar type III secretion

system (Aldridge et al. 2006). This bifunctionality of σ^{28} is an example of a dual role assumed by many type III secretion chaperones that often function both in facilitating secretion and as regulators of gene expression (Chilcott and Hughes 2000; Chevance and Hughes 2008). Another bifunctional secretion chaperone is FliT, the secretion chaperone for the flagellar cap protein FliD (Bennett et al. 2001) that also acts as an inhibitor of $FlhD_4C_2$ (Yamamoto and Kutsukake 2006; Aldridge et al. 2010). This dual role was suggested to couple completion of the hook-basal body complex and the concomitant start of FliD secretion with inhibition of class II and III gene expression, mediated by the released FliT (Yamamoto and Kutsukake 2006; Brown et al. 2008).

1.3.3.3 Regulation of expression of the flagellar master regulator operon *flhDC*

Flagellar gene expression is tightly regulated in response to a large array of environmental stimuli, including variations in temperature, osmolarity, pH and oxygen. Signal integration occurs mainly at the level of expression of the master regulator operon *flhDC* through both binding of numerous regulatory proteins to its promoter region and posttranscriptional control mechanisms (Soutourina and Bertin 2003; Pruss et al. 2006).

Flagellar synthesis is subject to carbon catabolite repression mediated by induction of *flhDC* expression upon binding of cAMP-CRP to the promoter region (Soutourina et al. 1999). H-NS binds to several sites upstream and downstream of the promoter, but *in vivo* exerts a positive effect on *flhDC* expression (Soutourina et al. 1999). In contrast, two LysR-type regulators, LrhA and HdfR were shown to negatively regulate *flhDC* expression by binding to sites in the promoter region and/or further downstream (Ko and Park 2000a; Lehnen et al. 2002). HdfR is negatively regulated by H-NS and therefore seems to be at least partly responsible for the positive effect that H-NS exerts on flagellar gene expression (Ko and Park 2000a). As $FlhD_4C_2$ seems to act as an autogenous repressor of its own operon, binding of the master regulator might add another factor to the nucleoprotein complex assembling at the *flhDC* promoter (Kutsukake 1997).

Moreover, several two-component systems are involved in regulating *flhDC* expression in response to environmental cues. The response regulator OmpR of the EnvZ/OmpR system binds to and represses expression from the *flhDC* promoter in its phosphorylated form, thus mediating regulation of flagellar gene expression in response to changes in osmolarity or in intracellular acetyl phosphate levels (Shin and Park 1995). The Rcs system negatively affects *flhDC* expression by binding of the response regulator RcsB together with its cofactor RcsA to a site downstream of the promoter (Francez-Charlot et al. 2003). The binding site partly

overlaps with one of the OmpR binding sites. The BarA/UvrY two-component system is involved in regulation of the RNA-binding regulator CsrA (Suzuki et al. 2002), and thereby might participate in post-transcriptional control of *flhDC* expression. As mentioned above, CsrA is a global post-transcriptional regulator that acts by binding to mRNA. CsrA affects mRNA stability and translation, in most cases in a negative fashion (Timmermans and Van Melderen 2010). In contrast, CsrA induces *flhDC* expression by binding to the *flhDC* mRNA and stabilizing it by an as yet unknown mechanism (Wei et al. 2001).

Other regulators shown to be involved in regulation of motility by unidentified mechanisms are the chaperones DnaJ, DnaK and GrpE (Shi et al. 1992), the replication initiation protein DnaA (Mizushima et al. 1994; Mizushima et al. 1997) and the nucleoid-associated protein HU (Nishida et al. 1997).

Further complexity is added by the observations that different *E. coli* strains and even different stocks of the same strain vary in the presence of insertion sequence elements in the region upstream of the *flhDC* promoter and that the presence of such an element correlates with increased motility (Barker et al. 2004). Interestingly, one of these insertion elements was recently shown to carry an σ^{54}-dependent promoter (Zhao et al. 2010). Under certain growth conditions, motility and flagellar gene expression seem to depend on σ^{54}, but this dependence does not necessarily require the presence of the σ^{54}-dependent promoter carried by the insertion element (Zhao et al. 2010; Dong et al. 2011). This involvement of σ^{54} is interesting considering that the regulatory cascades controlling motility in many polarly flagellated bacteria depend on σ^{54} (McCarter 2006).

In addition to major signal integration at the level of *flhDC* expression, the more than 60 members of the flagellar regulon represent additional known or potential targets for transcriptional, post-transcriptional and post-translational mechanisms altering flagellar synthesis and function.

Comparison of the regulators affecting *flhDC* expression with those that influence expression of the curli regulator CsgD (see 3.2) shows a large overlap, indicating the presence of a complex network coordinating motility and adhesion and the transition between the two lifestyles (Pruss et al. 2006). However, many details of this regulation, concerning e.g. the role of antagonistic versus synergistic control of the two lifestyles by some of these regulators and the identity and integration of additional regulators in this network still remained unknown when this work had begun.

2. Aims

In studies preceding this work, the Hengge group has used the non-motile *E. coli* K12 strain MC4100, which carries a frameshift mutation in the *flhDC* operon, for the study of curli fimbriae regulation (Weber et al. 2006). However, observations in other *E. coli* strains suggested that curli fimbriae expression may be coordinated with motility, resulting in a temporal succession of motility and curli fimbriae expression in cells grown in rich medium: Motility peaks in post-exponentially growing cells and subsequently declines, while curli fimbriae expression is induced upon entry into stationary phase. (Adler and Templeton 1967; Amsler et al. 1993; Arnqvist et al. 1994). This growth phase-dependent succession of the two lifestyles was a first indication for a mutual exclusion and inverse coordination of motility and curli fimbriae-mediated adhesion. Such an inverse coordination seems to be a logic prerequisite for both optimal swimming and successful adhesion, since binding to surfaces should interfere with motility, while rotating flagella are likely to impede permanent adhesion. Thus, a precise coordination of the two systems appears to be essential in the transition from the planktonic to the adhesive lifestyle, which also represents the initial step in biofilm formation.

These observations and considerations constituted the basis for the work presented in this thesis, a major aim of which was the identification and detailed characterization of regulatory processes involved in the coordination of motility and curli fimbriae-mediated adhesion in *E. coli*. The emphasis was put on an analysis of the influence that the flagellar system exerts on curli fimbriae expression. To this end, a system that artificially induces flagellar gene expression in the non-motile strain MC4100 was used as a starting point to uncover members of the flagellar gene regulon that are directly involved in communication with the curli control network and to analyse the regulatory level on which this communication takes place. The motile strain W3110, which shows the growth phase-dependent succession of motility and adhesion, should subsequently be used to characterize the role that the identified regulatory links between the two systems play in the switch between motility and adhesion.

These studies took place in parallel with other studies of the Hengge group that used different approaches to identify additional levels of communication between the cascades controlling curli fimbriae expression and flagellar motility. The final goal of these analyses was to integrate all data into a comprehensive model of the regulatory processes that direct the lifestyle-switch from motile-planktonic to sedentary-adhesive cells.

Amongst others, the studies performed in the course of this work led to the identification of the flagellar regulator FliZ, which contributes to the coordination of motility and adhesion by acting as a potent inhibitor of the σ^S regulon, which includes curli fimbriae expression. The comprehensive effect that FliZ exerts on σ^S-dependent gene expression made this regulator the focus of our interest and a large part of this thesis was aimed at a detailed *in vivo* and *in vitro* characterization of the molecular mechanism of action of this novel sigma factor antagonist.

Since several groups have observed FliZ-dependent effects on motility in *E. coli* (Mytelka and Chamberlin 1996; Girgis et al. 2007) and *Salmonella* (Ikebe et al. 1999; Kutsukake et al. 1999; Saini et al. 2008), without elucidating the underlying mechanisms, an analysis of the role that FliZ plays in the regulation of motility in *E. coli* was also included into this work.

At the beginning of this thesis, several important details on the regulation of curli fimbriae expression had remained elusive. These concerned, for example, the precise mode of operation of the YdaM/YciR-c-di-GMP control module that regulates *csgD* transcription, and the identity of the involved c-di-GMP effector. Moreover, the exact mechanisms through which other important regulators, such as H-NS, affect curli expression had not been clarified.

On the one hand, answers to many of these open questions were of general interest since they promised to provide interesting new insights into the general principles of c-di-GMP signalling. On the other hand, elucidation of some of these points also seemed to be relevant for a better understanding of the mechanisms that contribute to the coordination of motility and curli fimbriae expression. Therefore, the clarification of some of these open questions concerning regulation of curli fimbriae expression was another goal of this thesis.

3. Materials and Methods

3.1 Chemicals, materials and technical equipment

The standard chemicals used in this work were purchased from the following companies: AppliChem, Difco, Millipore, Merck, Roth, Sigma-Aldrich, VWR. The suppliers of additional materials and technical equipment used are listed in table 3.1 and/or indicated in the respective methods parts of this chapter.

Tab. 3.1: Suppliers of materials and technical equipment used in this work.

Materials	
Product	**Supplier**
Acrylamide-Bisacrylamide	Roth
Agarose	Biozym
Alkaline phosphatase: Calf Intestinal Phosphatase (CIP)	New England Biolabs
Antibiotics	Roth, Sigma Aldrich
Anti-Rabbit IgG-Alkaline Phosphatase antibody	Sigma-Aldrich
Anti-Strep antibody	USBiological
APS (Ammonium persulfate)	Roth
BCIP (5-Bromo-4-chloro-3-indolyl phosphate)	Roth
Blotting membrane for proteins: Roti-PVDF, pore size 0.45µM	Roth
Blotting paper	Roth, Whatman
Bradford-Solution for protein determination	AppliChem
Bromphenol blue	Roth
Chitin-Beads	New England Biolabs
Coomassie Brilliant Blue R-250	AppliChem
DNA preparation kit: QIAamp DNA Mini Kit	Analytik Jena
DNaseI for protein purification buffer (powder)	Roche
DNA size markers: 100 bp DNA Ladder, λDNA-B*st*EII Digest	New England Biolabs
DNA polymerase: Phusion	Biozym
DNA polymerase: Opti-Taq	Roboklon
DNA polymerase: Vent	New England Biolabs
dNTPs Mix	MP Biomedicals
Gel extraction kit	Qiagen
IPTG (isopropyl-β-D-1-thiogalactopyranoside)	Roth
Milk powder	Roth
NBT (Nitroblue tetrazolium chloride)	AppliChem
Oligonucleotide primers	Metabion
ONPG (ortho-nitrophenyl-β-D-galactopyranoside)	Roth, Serva
PCR purification kit	Qiagen
Plasmid mini preparation kit: QIAprep Spin Miniprep Kit, InnuPREP Plasmid Mini Kit	Qiagen, Analytik Jenal
Protein size marker: Prestained Protein Marker	New England Biolabs
Restriction enzymes	New England Biolabs
T4 DNA ligase	New England Biolabs
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Roth
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	AppliChem
Xylene Cyanole	Roth

Tab. 3.1 (continued)	
Technical equipment	
Device	**Supplier**
Äkta system	GE Healthcare
Centrifuges	Eppendorf, Heraeus, Thermo Scientific
Electroporator	BioRad
ELISA-Reader	BioRad
French Press	Thermo Electron Corporation/Amincon SLM Instruments
Gel documentation	Alpha Innotech/ Biozym
Gel electrophoresis	BioRad, Peqlab, Thermo Scientific, C.B.S. Scientific CO
Gel filtration column: Superdex 75 (16/60)	GE Healthcare
Incubators	Infors HT
Microarray Scanner: GenePix 4100A	Molecular devices (Axon)
NanoDrop	Peqlab
PCR-Thermocycler	MWG Biotech, Biometra
PhosphoImager (FLA-2000G)	FujiFilm
Photo developing machine	Protec
Photometer	Pharmacia Biotech
ytRNA (yeast carrier tRNA)	Roche

3.2 Media and Additives

3.2.1 Media

For cultivation in nutrient-rich medium, Luria-Bertani (LB) medium, containing 5 g of yeast extract, 10 g of Bacto-tryptone and 5 g of NaCl per litre, was used (Silhavy et al. 1984).
For LB/agar plates 15 g of Bacto-Agar per litre LB medium was added.
Motility plates contained 5 g of Bacto-tryptone, 5 g of NaCl and 3 g of Bacto-agar per litre.

3.2.2 Additives

The following media additives were prepared as highly concentrated stock solutions.

Tab. 3.2: Additives and their final concentrations in media.

Additive	**Dissolvent**	**Final concentration** (if not stated otherwise)
Ampicillin	water	100 µg/ml
Chloramphenicol	70% ethanol	15 or 30 µg/ml
Kanamycin	water	50 µg/ml
Sodium citrate	water	20 mM
Tetracylin	70% ethanol	5 µg/ml
X-Gal	DMF (dimethylformamide)	30 µg/ml

3.3 Microbiological methods

3.3.1 Sterilization

Media and chemical solutions were wet-autoclaved for 20 minutes at 120°C and 1 bar, glassware was dry-heat-sterilized at 180°C for 8 hours and heat-sensitive solutions were sterilized by filtration.

3.3.2 Long-term storage of bacterial strains

For long-term storage of bacterial strains, overnight cultures of bacterial strains grown in LB medium were mixed with DMSO (dimethyl sulfoxide) at a final concentration of 7 % and stored at -80°C.

3.3.3 Growth conditions

Liquid cultures were grown at 28°C or 37°C in glass flasks or tubes filled up to 20 % of the total volume. Flasks were incubated in a water bath with shaking at 300 rpm to allow for aeration, cultures in tubes were aerated by rolling. Bacteria grown on solid medium were incubated at 28°C or 37°C. For standard experiments bacterial cultures grown in LB medium were inoculated to a start OD_{578nm} of 0.05.

3.3.4 Bacterial motility assay

To test for motility, 3 µl of overnight cultures adjusted to an OD_{578nm} of 2 or 3 were inoculated into motility plates (see 3.2.1) and allowed to grow and swim for 3-8 hours at 28°C or 37°C.

3.3.5 Determination of the cell density of liquid bacterial cultures

The cell density of liquid bacterial cultures was determined by measuring the optical density at a wavelength of 578nm, using sterile medium as a reference. For cultures with an $OD578_{nm}$ higher than 0.5, probes were diluted before measuring to ensure a linear correlation between the optical density and the bacterial concentration.

3.3.6 Transformation

Transformation denotes the uptake of free DNA by bacterial cells. Electroporation and TSS transformation are two forms of transformation used to transfer plasmid DNA into bacterial cells. Electroporation (Calvin and Hanawalt 1988) was used here to transfer plasmid DNA generated in ligation mixtures into bacterial cells, since it has a high transformation efficiency. Electrocompetent cells were generated by a standard protocol (Sambrook et al. 1989). Purified plasmids were transferred into bacterial strains by the less efficient, but faster TSS transformation (Chung et al. 1989).

3.3.7 P1 transduction

To generate a P1 lysate of a bacterial strain, 5 ml of LB medium were inoculated with 50 µl of an overnight culture of this strain and cells were grown at 37°C under aeration to an OD_{578nm} of approximately 0.3. After addition of one drop of 1M $CaCl_2$ and a few drops of the wild-type $P1_{vir}$ lysate, cells were incubated for 3-8 hours at 37°C with aeration until cells were sufficiently lysed. To kill residual cells, 5-10 drops of chloroform were added, the mixture was vortexed and cell debris was removed by centrifugation. The supernatant contained the P1-Phages that, in addition to their own DNA, carry host cell DNA fragments together covering the entire chromosome of the bacterial strain. 5 drops of chloroform were added to the supernatant and it was stored in sterile glass tubes at 4°C.
A standard protocol was used to generate bacterial strains by P1 transduction (Miller 1972; Miller 1992).

3.4 Molecular biological and biochemical methods

3.4.1 Determination of DNA concentrations

The absorbance of a DNA solution was determined at 260 nm and the concentration was calculated considering that a DNA solution with an OD_{260nm} of 1 contains 50 µg/ml RNA.

3.4.2 Polymerase chain reaction (PCR)

PCR was performed according to standard protocols (Sambrook et al. 1989) using the DNA polymerases Phusion (Biozym), Opti-Taq (Roboklon) and Vent (New England Biolabs) and the primers listed in table 3.6 at the end of this chapter. Primers were synthesized by Metabion (Martinsried). PCR products were analyzed on 1 % or 1.5 % agarose gels. PCR-fragments used for cloning into plasmids and for EMSA and DNaseI footprint assays were purified from agarose gels using the QIAquick gel-extraction kit (Qiagen).

3.4.3 Recombinant PCR (two step-four primers method)

In order to generate PCR fragments in which single or a few nucleotides have been exchanged compared to the template DNA, a two-step PCR protocol was used (Higuchi 1990). Four primers were designed: two *outside primers* that bind to the upstream and downstream end of the final PCR product and two *inside mutagenesis primers* with complementary sequences that contain the desired mutation(s) in the middle of their sequences. Using appropriate combinations of outside and inside mutagenesis primers, two PCR products with an overlapping sequence that contains the nucleotide exchanges were produced and purified by agarose gel electrophoresis and subsequent extraction. Together, these PCR fragments were used as templates for a second PCR with the two outside primers. In this PCR, the two overlapping sequences of the PCR products anneal and the recessed 3´-ends were extended by DNA polymerase yielding a DNA duplex spanning the entire region between the outside primers. This annealing was favoured by low annealing temperatures (45-48°C) and increased annealing times during the first 5 PCR cycles. The resulting DNA molecule, which carries the desired mutations, was amplified in subsequent PCR cycles.

3.4.4 Preparation of chromosomal DNA and plasmid DNA

Chromosomal DNA was prepared using the QIAamp DNA Mini Kit (Qiagen). Plasmid DNA was prepared from bacterial cells using the QIAprep Spin Miniprep Kit (Qiagen) or the InnuPREP Plasmid Mini Kit (Analytik Jena).

3.4.5 Agarose gel electrophoresis

DNA was analyzed on 1-1.5 % agarose gels (in TAE buffer (40 mM Tris (trishydroxyaminomethane), 1 mM EDTA, 20 mM acetic acid). DNA samples were mixed with 6x DNA sample buffer (0.25 % bromphenol blue, 0.25 % xylene cyanole, 30 % glycerol) and loaded in parallel with DNA size markers (100 bp DNA Ladder, λDNA-B*st*EII Digest, New England Biolabs). Gels were run at 90-120 V and were subsequently stained with ethidium bromide (0.5 µg/ml).

3.4.6 Plasmid construction

The plasmids constructed in this study are listed in table 3.5 at the end of this chapter.

3.4.6.1 Restriction digest and ligation

For standard plasmid constructions, DNA-fragments amplified by PCR using the primers listed in table 3.6 at the end of this chapter were digested with restriction enzymes (New England Biolabs) according to the manufacturer´s instructions. In order to prevent religation of plasmid DNA, digested plasmids were dephosphorylated by incubation with calf intestinal phosphatase (CIP, New England Biolabs) for 1 h at 37°C. Digested DNA fragments were purified by agarose gel electro-phoresis and subsequent extraction using the QIAquick gel-extraction kit (Qiagen) or with the QIAquick PCR purification kit (Qiagen) and were ligated with plasmid DNA using T4 DNA ligase (New England Biolabs). Ligation reactions were performed for 2 hours at 20°C or overnight at 4°C.

3.4.6.2 Construction of the small RNA plasmid pRyeB

The pRyeB plasmid was constructed according to the protocol for construction of small RNA plasmids described by Urban and Vogel (Urban and Vogel 2007). A DNA fragment obtained after PCR using plasmid pZE12-*luc* as a template and primers PLlacOB and PLlacOC (tab. 3.6) was digested with *Xba*I yielding two fragments. The larger fragment, which, amongst others, carries

the p_{LlacO} promoter and a strong *rrnB* terminator, was ligated to a PCR fragment produced using the primers listed in table 3.6 and W3110 wild-type DNA as template DNA. This fragment carries the *ryeB* gene starting at the transcriptional start site with a 5´monophosphate generated at this site by using a phosphorylated primer, and a *Xba*I restriction site introduced by the downstream primer, which was cut by a *Xba*I digest prior to ligation. In the resulting plasmid pRyeB, the transcriptional start site of the *ryeB* gene is ligated to position -1 of the p_{LlacO} promoter. Thus, expression of *ryeB* from this plasmid produces a transcript identical to the one produced from the chromosomal copy of this gene.

3.4.6.3 Electroporation

Ligated plasmid DNA was transferred into bacterial cells by electroporation (see 3.3.6) using 1-2 µl of the ligation mixture and 50 µl of electrocompetent cells.

3.4.6.4 Sequence confirmation

Plasmids were isolated from bacterial cells and plasmids containing inserts of the correct size were identified by restriction digests and sent to Agowa (now LGC Genomics, Berlin) or GATC (Konstanz) for sequencing of the insert and adjacent vector parts.

3.4.7 Construction of chromosomal *lacZ* fusions

The primers used for the construction of *lacZ* fusions are listed in table 3.6 at the end of this chapter. The PCR fragments were cloned into the translational fusion vector pJL28 (Lucht et al. 1994) or into the transcriptional fusion vector pCAB6 (Barembruch and Hengge 2007). Since many of the translational fusions used here only contain a few codons of the open reading frame of the respective gene, they mainly reflect transcription of the corresponding gene and do not monitor post-transcriptional control mechanisms that are based e.g. on mRNA structures. *lacZ* fusions were transferred to the bacterial chromosome as described by Simons et al. (Simons et al. 1987). The plasmid-encoded *lacZ* fusions were transferred into phage λRS45 or λRS74 by *in vitro* recombination between the plasmid-encoded *bla* and *lacZ* genes and the phage-encoded *bla´* and *lacZ´* genes. Stable integration of the *lacZ* fusions into the chromosome was subsequently achieved by integration of the phages at the bacterial *attB*-site. In order to exclude strains that have integrated multiple copies of the phage, single lysogeny was tested by PCR as described by Powell et al. (Powell et al. 1994).

3.4.8 Construction of mutants

Deletion-insertion mutations and non-polar in-frame deletion mutations, obtained after elimination of the antibiotic resistance cassette, were generated as described by Datsenko and Wanner (Datsenko and Wanner 2000) using the primers listed in table 3.6 at the end of this chapter.

3.4.9 Determination of β-galactosidase activity

β-galactosidase activity was assayed according to Miller (Miller 1972), using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate and is reported as µmol of *o*-nitrophenol per min per mg of cellular protein. Experiments showing the expression of *lacZ* fusions along the entire growth cycle were done at least twice and a representative experiment is shown.

3.4.10 RNA preparation

For Northern blot analysis, primer extension analysis and DNA microarray analysis, RNA was prepared by hot phenol/chloroform extraction. To this end, an appropriate volume of a bacterial culture (30 ml for microarray experiments, 5 ml for Northern blot analysis and primer extension analysis) were harvested, mixed with ice-cold RNA-stop solution (5 % phenol in ethanol; 3.5 ml added to samples taken for microarray experiments and 0.63 ml added to samples taken for Northern blot analysis and primer extension analysis) and incubated on ice for 5 minutes. After centrifugation at 4°C, the cell pellet was stored at -80 °C.

Cell lysis and nucleic acid extraction were performed as described by Tani et al. (Tani et al. 2002). DNA was digested with 10 µl of RNase-free DNaseI (10 u/µl, Roche/Boehringer Mannheim) for 20 min at 37°C. RNA was successively extracted in 1 volume of Aqua-PCI (phenol:chloroform:isoamyl alcohol, 25:24:1, pH 4.5-5) and 1 volume of CI (chloroform:isoamyl alcohol, 24:1), followed by at least 30 minutes of precipitation at -80°C upon addition of 0.1 volumes of 3 M sodium acetate (pH5.2) and 3 volumes of ice-cold 100 % ethanol. The RNA was pelleted by centrifugation at maximal speed at 4°C and the pellet was washed with 80 % ice-cold ethanol, pelleted again and air-dried at room temperature. The RNA was resuspended in 50 µl of RNase-free water.

The absorbance of the RNA solution was determined at 260 nm and the concentration was calculated considering that a RNA solution with an OD_{260nm} of 1 contains 40 µg/ml RNA.

Purity of the RNA was checked by measuring the absorbance of the RNA solution at 280nm (detection of protein impurities) and 230 nm (detection of phenol impurities). Moreover, the RNA was checked for DNA impurities and possible degradation by denaturing agarose gel electrophoresis or agarose gel electrophoresis with subsequent ethidium bromide staining.

3.4.11 Electrophoretic separation of RNA by denaturing agarose gel electrophoresis

To control the quality of RNA preparations and for Northern blot analysis of larger RNA molecules (e.g. *fliAZY* mRNA), RNA was separated on denaturing agarose gels [1.2 g agarose, 72 ml DEPC-water, 10 ml 10x MOPS buffer (0.4 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA, 0.1 % DEPC), 18 ml 37 % formaldehyde]. 20 µg of RNA in 10 µl of water were mixed with 15 µl of RNA loading buffer (250 µl of deionized formamide, 83 µl 37 % formaldehyde, 50 µl 10x MOPS, 4 µl 1 % bromphenol blue, 13 µl water), heated for 15 minutes at 65°C, cooled down on ice for 5 minutes and loaded into the dry pockets of the gel placed in 1x MOPS buffer. 10 µl of RNA molecular weight marker I (Roche) mixed with 15 µl of RNA loading buffer were heated and loaded in parallel. The run was started at 100 mA until the samples had entered the gel matrix and was continued at 80V after the gel had been covered with 1x MOPS buffer. The gel was run for 3-4 hours and was either stained with ethidium bromide (for RNA quality control) or the RNA was subsequently transferred to a membrane by Northern blot.

3.4.12 Northern blot analysis

RNA transfer after electrophoretic separation of RNA in denaturing agarose gels

After RNA had been separated in denaturing agarose gels, the formaldehyde was removed by rinsing the gel several times in water and soaking it twice in 20x SSC (3 M NaCl, 0.3 M sodium citrate, DEPC, pH 7.0) for 15 minutes. The RNA was transferred to a positively charged nylon membrane (Roche), which had been pre-equilibrated in water and 10x SSC, by semi-dry vacuum-blotting in 10x SSC at approximately 5 Hg for 1.5 hours. The membrane was washed in 2x SSC, the RNA was UV-cross-linked to the membrane for 4 minutes and the successful transfer was checked by staining with methylene blue solution (0.02 % methylene blue, 0.3 % sodium acetate (pH 5.5), DEPC). Methylene blue was removed by washing the membrane twice in bleaching buffer (0.2x SSC, 1 % SDS, DEPC) for 15 minutes and twice in 2x SSC for 5 minutes.

Separation and transfer of RNA using denaturing polyacrylamide gels

For the analysis of smaller RNA molecules (e.g. *fliZ* mRNA expressed from pCAB18, *ymgB-Strep* mRNA), RNA was separated on denaturing 4.5 % polyacrylamide gels containing 7 M urea [42 g urea, 10 ml 10x TBE (108 g Tris, 55 g boric acid, 40 ml 500 mM EDTA (pH 8.0) per litre), 11.25 ml Rotiphorese Gel40 (Roth; acrylamide:bisacrylamide = 19:1), 50 µl TEMED, 500 µl 10 % APS in a total volume of 100 ml], using a gel electrophoresis system from C.B.S. Scientific CO. 5 µg of RNA in 5 µl of water were mixed with 5 µl of gel loading buffer II (Ambion), heated to 75°C for 5 min and loaded in parallel with 3 µl of RNA molecular weight marker III (Roche) that had been mixed with 2 µl of water and 5 µl of gel loading buffer II (Ambion). The gel was run for 1.5-2 hours at 200 V. The RNA was transferred onto a positively charged nylon membrane (Roche) by 3 hours of electro-blotting at 500 mA in a tank blotter (Peqlab), and was UV cross-linked to the membrane for 4 minutes.

Detection of specific RNA molecules

RNA molecules of interest were detected with a DIG-labelled DNA probe. The probe was generated by PCR using a PCR-DIG labelling mix (Roche), the primers listed in table 3.6 at the end of this chapter and 40 ng of unlabelled gel-purified PCR fragments (generated with the same primers) as DNA templates. After pre-hybridisation in DIG Easy Hyb hybridisation solution (Roche) at 47°C for 1 hour, 10-20 µl of the DIG-labelled probe in a total volume of 50 µl were denatured at 95°C for 5 minutes, cooled on ice for 5 minutes, added to the hybridization solution and incubated overnight with the membrane at 47°C. To remove unbound probe, the membrane was washed twice with washing buffer 1 (2x SSC, 0.1 % SDS, DEPC) at 42°C for 5 minutes and twice with washing buffer 2 (0.1 % SSC, 0.1 % SDS) at room temperature for 30 minutes. The membrane was rinsed with detection buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and after 30 min of incubation in blocking buffer (detection buffer 1 containing 1 % blocking reagent (Roche)) an anti-Digoxigenin-alkaline phosphatase antibody (Roche) was added at a dilution of 1:10 000 and incubated with the membrane for 1 hour. The membrane was washed twice with detection buffer 1 for 15 minutes to remove excess antibody and was then equilibrated in detection buffer 2 (0.1 M Tris (pH 9.5), 0.1 M NaCl, 0.05 M $MgCl_2$) for 2 minutes before it was covered with CDP-Star solution (Roche) in the dark (CDP-Star is an ultra-sensitive chemiluminescent substrate for alkaline phosphatase). After 5 min of incubation, the CDP-Star solution was removed, the membrane was covered with foil and the chemiluminescent signal was detected with a

chemiluminescent detection film (Roche), which was exposed to the membrane for 5-30 minutes.

3.4.13 Microarray analysis

For the microarray analysis, *Escherichia coli* K-12 microarray slides produced at the Max-Planck-Institut für Infektionsbiologie in Berlin (AG Mollenkopf) were used. These microarray slides carry 4288 gene-specific 50mer oligonucleotide probes, synthesized by MWG (Ebersberg) that represent all open reading frames in the *E.coli* genome.

RNA was prepared from MC4100 derivatives carrying either pFliZ or the empty vector pCAB18. Cells were grown in LB medium supplemented with ampicillin at 28°C without inducer and harvested at an OD_{578nm} of 4. Equal amounts (50µg) of total RNA from these two strains were used to synthesize cDNA labelled with the fluorescent nucleotide analogue Cy3-dUTP or Cy5-dUTP (Amersham) as described by Weber et al. (Weber et al. 2005). The labelled cDNA was purified with the illustra CyScribe GFX purification kit (GE Healthcare) according to the manufacturer`s instructions. The two labelled probes were mixed, the volume of the mixture was reduced to approximately 10 µl by evaporation in a vacuum centrifuge and after addition of 6 µl of water the mixture was heated at 95°C for 5 minutes. After 1 minute of incubation on ice, 35 µl of SlideHyb^{TM}-buffer (Ambion), pre-heated to 68°C, were added and the mixture was applied to the microarray slide. The microarray was hybridized for 20-24 hours at 45°C. The washing procedure, fluorescence detection and image analysis were carried out as described by Weber et al. (Weber et al. 2005) using a GenePix 4100A (Molecular devices (Axon)) laser scanner and the software GenePix Pro 4.1. Microarray experiments were repeated two times (biological replicates) with a dye-swap. Genes were considered differentially regulated when (i) signal-to-noise ratios exceeded a factor of three, (ii) the sum of median intensity counts were above 300 and (iii) relative RNA level differences (ratios) were at least threefold in both of the two independent experiments.

3.4.14 Primer extension analysis

Primer extension analysis was carried out to determine the transcriptional start site of the *yciR* gene. Total RNA was prepared as described above (3.4.10) from W3110 wild-type cells, *yciR::kan* mutant cells (NS49) and W3110 wild-type derivatives carrying plasmid pCP5 (see tab. 3.5) grown in LB medium at 28°C to an OD_{578nm} of approximately 3.5.

The primer listed in table 3.6 at the end of this chapter was labelled with γ-^{32}P-ATP in a reaction mixture containing 2 µl of the primer (10 pmol/µl), 1 µl of T4 polynucleotide kinase (PNK, 10 u/µl, Fermentas), 1.5 µl of T4 PNK-buffer (Fermentas), 2 µl of γ-^{32}P-ATP (Hartmann Analytic) and 8.5 µl of water. After incubation at 37°C for 60 min, the T4 PNK was inactivated at 90°C for 3 minutes and the labelled primer was purified via a Sephadex G-25 column (GE Healthcare) according to the manufacturer´s instructions.

2 µl of the labelled primer were incubated with 10 µg of RNA in 11 µl of water and 1 µl of RNase-free dNTPs mix (10mM, Invitrogen) at 65°C for 5 minutes. After 5 minutes of incubation on ice, 1 µl of Superscript III reverse transcriptase (Invitrogen), 4 µl of 5x First Strand Buffer (Invitrogen) and 1 µl of DDT (0.1 M, provided with Superscript III reverse transcriptase) were added and the mixture was incubated at 45°C for 60 minutes. The enzyme was inactivated by incubation at 70°C for 15 minutes.

As a reference, a DNA sequence ladder was generated with the same labelled primer using the CycleReader DNA Sequencing Kit (Fermentas). The sequencing reactions were performed according to the manufacturer´s instructions using 1.5 µl of the labelled primer and 100 fmol of a template DNA fragment generated with the unlabeled primer and the forward primer listed in table 3.6 at the end of this chapter.

2 µl of the primer extension reactions mixed with 5 µl of Stop-Solution from the CycleReader DNA Sequencing Kit and 3 µl of each sequencing reaction mixed with 4 µl of 1:4-diluted Stop-Solution were heated at 90°C for 3 minutes and loaded onto a denaturing 6 % polyacrylamide sequencing gel containing 7 M urea (42 g urea, 10 ml 10x TBE, 15 ml Rotiphorese Gel40, 50 µl TEMED, 500 µl 10 % APS in a total volume of 100 ml). The gel had been pre-warmed by a pre-run at 60W for 1 hour. Samples were run for approximately 2 hours at 60W and the gel was vacuum-dried for 90 minutes before being autoradiographed using a FLA-2000G Imager (FujiFilm).

3.4.15 Extraction of proteins from bacterial culture samples

In order to extract proteins from bacterial culture samples, LB medium was removed by centrifugation and cells were resuspended in M9 medium (6 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.5 g NaCl, 0.1 g NH_4Cl per litre in water) with 10 % trichloroacetic acid (TCA) and incubated on ice for 30 minutes for protein precipitation. After centrifugation, precipitated protein was washed with ice-cold acetone, which was subsequently removed by centrifugation. The protein pellet was dried at room temperature and resuspended in SDS-sample buffer (0.06 M Tris (pH 6.8), 2 % SDS, 10 % glycerol, 3 % β-mercaptoethanol, 0.005 % bromphenol blue).

To standardize the protein concentration, the following correlation between the OD_{578nm} and the protein concentration of a bacterial culture was used to calculate the amount of SDS-sample buffer required to establish a concentration of 1 µg whole cell protein per µl: 1 ml of a cell suspension at an OD_{578nm} of 1 contains approximately 107 µg of protein (Regine Hengge-Aronis, unpublished results).

3.4.16 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

In SDS-PAGE, a polyacrylamide matrix is used to separate proteins according to their sizes. Binding of SDS (sodium dodecyl sulphate) to proteins results in denaturation and conveys a negative charge. Thus, the natural charge of the protein does no longer influence the separation process. SDS page was carried out according to standard protocols (Laemmli 1970; Sambrook et al. 1989) in a Mini-PROTEAN II cell (Biorad). The polyacrylamide gel consisted of a lower 12 % polyacrylamide gel [2.5 ml LT buffer (36.34 g Tris, 0.8 g SDS in 200 ml water; pH 8.8), 4 ml Rotiphorese Gel 30 (Roth; acrylamide:bisacrylamide = 37.5:1), 3.45 ml water, 5 µl TEMED, 5 µl 10 % APS] and an upper 4 % polyacrylamide gel [1.25 ml UT buffer (6.06 g Tris, 0.8 g SDS in 100 ml water; pH6.8), 0.65 ml Rotiphorese Gel 30, 3.07 ml water, 5 µl TEMED, 25 µl 10 % APS]. Protein samples in SDS-sample buffer were heated to 100°C for 10 min prior to loading and gels were run at 25 mA per gel in SDS-PAGE buffer (25 mM Tris, 0.19 M glycerol, 0.1 % SDS). After electrophoresis, polyacrylamide gels were either stained with coomassie to visualize proteins or the proteins were transferred onto a membrane for immunoblot analysis.

3.4.17 Coomassie staining

Coomassie Brilliant Blue R-250 (AppliChem) was used to stain proteins separated in polyacrylamide gels. The gels were heated in staining solution (25% isopropanol, 10 % acetic acid, 0.05 % Coomassie Brilliant Blue R-250) until boiling, then incubated with shaking for 5 minutes and destained in 10 % acetic acid until protein bands were clearly visible.

3.4.18 Immunoblot analysis (Western blot)

Immunoblot analysis was used for the detection of specific proteins. After SDS-PAGE proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a Mini Trans-Blot cell (Biorad). Prior to the transfer, the membrane was successively equilibrated in methanol, water and transblot buffer (25 mM Tris, 192 mM glycine, 20 % methanol). The

polyacrylamide gel and the membrane were placed between blotting paper and foam pads in cold transblot buffer and the protein transfer was performed for 1 hour at 100 V.

After blotting, the membrane was blocked in TBSTM [TBST buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 0.05 % Tween-20) with 5 % milk powder] for at least 30 minutes. The primary antibody (all primary antibodies used here had been produced in rabbits) was added (3 µl in 20 ml TBSTM for the anti-CsgD, anti-FliZ and anti-σ^S antibodies and 4 µl in 20 ml TBSTM for the anti-Strep antibody) and incubated with the membrane for 2 hours with shaking. The membrane was washed three times with TBST buffer for 10 minutes and was then incubated in TBSTM with the secondary antibody (goat-anti-rabbit-alkaline phosphatase conjugate, Sigma-Aldrich, 3 µl in 20 ml TBSTM) for one hour. After washing in TBST for 10 minutes and equilibration in AP (alkaline phosphatise) buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$) for another 10 minutes, the Western blot was developed in 10 ml AP buffer by addition of a chromogenic substrate of alkaline phosphatase [33 µl BCIP solution (50 mg/ml in dimethylformamide) and 66 µl NBT solution (50 mg/ml in 70 % dimethylformamide)]. The catalysis of the colour-forming reaction was stopped by washing the membrane in water and the blot was dried on blotting paper. The quantification software Image Gauge was used to quantify the scanned or photographed Western blots, considering background intensities as well as intensities of reference bands where possible.

3.4.19 Protein over-expression and purification

3.4.19.1 Preparation of FliZ under denaturing conditions

For the generation of antibodies against FliZ, the protein with a N-terminal 6xHis-tag was purified under denaturing conditions. However, over-expression of 6xHis-FliZ was problematic, since cells carrying the pQE30-XA-FliZ plasmid grew extremely slowly, indicating that over-expression of FliZ was toxic to the cells. Moreover, cells transformed with this plasmid frequently did not show any over-expression of 6xHis-FliZ, which was most likely due to generation of suppressor mutations. Therefore, over-expression was tested in 5 ml overnight cultures prior to inoculation into larger culture volumes and successful over-expression was confirmed again after cells had been harvested.

3 litres of LB medium, supplemented with ampicillin and kanamycin, where inoculated with positively tested over-night cultures of FI1202 cells carrying plasmid pQE30-XA-FliZ and the helper plasmid pRep4, which constitutively expresses the LacI repressor. Over-expression was induced after growth for approximately 8 h at 28°C by addition of 1 mM IPTG and cells were harvested by centrifugation after overnight growth at 28°C. After

successful over-expression of 6xHis-FliZ had been confirmed by SDS-PAGE, the purification was performed according to the QIAGEN protocol for preparation of 6xHis-tagged proteins under denaturing conditions: The cell pellet (wet weight 4.2 g) was resuspended in 21 ml of buffer B (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea; pH 8) and stirred at room temperature for 2 hours. Due to insufficient cell lysis, the suspension was subsequently subjected to sonification. The cellular debris was then removed by centrifugation. The supernatant was mixed with 4 ml of Ni-NTA agarose slurry (Qiagen), pre-equilibrated in buffer B and incubated with shaking for 1 hour. Unbound protein was removed by washing with 2 litres of buffer C (100 mM NaH_2PO_4, 10 mM Tris, 8M urea; pH 6.3) and 6xHis-FliZ was eluted in buffer E (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea; pH 4.5). Samples taken at each step of over-expression and purification were analyzed by SDS-PAGE.

3.4.19.2 Preparation of FliZ and FliZ-R108A under native conditions

Due to the above-described problems with over-expression of 6xHis-FliZ from plasmid pQE30-XA-FliZ, plasmid pTYB12-FliZ was constructed that expresses FliZ as a fusion protein carrying a N-terminal self-cleavable intein tag. The intein tag, which carries a chitin-binding domain, is a large tag (55 kDa) that can “hide” parts of the protein fused to it, which can reduce toxicity during over-expression. Consistently, the above-described problems did not occur when FliZ was over-expressed from this plasmid.

For purification of FliZ and FliZ-R108A under native conditions, plasmids pTYB12-FliZ and pTYB12-FliZ-R108A were transformed into strain ER2566, cells were grown at 28°C in 6 litres of LB medium supplemented with ampicillin and over-expression was induced by addition of 0.5 mM IPTG at an OD_{578} of approximately 0.8. The culture was then transferred to 16°C to increase the solubility of the over-expressed proteins and cells were harvested the next day. The cell pellets were resuspended in approximately 30 ml of buffer A (500 mM NaCl, 20 mM Tris (pH 8), 0.1 % Triton X 100, trances of DNaseI powder) and cells were lysed using a French Press. Cell debris was removed by centrifugation and the supernatant was incubated for 1 hour with approximately 4 ml of chitin beads pre-equilibrated in buffer B (500 mM NaCl, 20 mM Tris (pH 8)). After washing with 200-300 ml of buffer B and equilibration with 3-4 ml of buffer C (500 mM NaCl, 20 mM Tris (pH 8), 50mM DTT) the chitin beads with bound protein were incubated in buffer C over night at 16-18°C. During this incubation, the high DTT concentration in buffer C induced a self-cleavage reaction that released the protein from the chitin-bound intein tag. The cleaved protein carries three extra amino acids at the N-terminus. The proteins were eluted the next day with buffer B and

collected in 1 ml fractions. Fractions containing protein were determined using Bradford solution (AppliChem), pooled and run over a Superdex 75 (16/60) column (GE Healthcare), using an Äkta System. The run was performed at 1 ml per minute using buffer D (500 mM NaCl, 20 mM Tris (pH 8), 0.2 mM DTT). The eluate was collected in 2 ml fractions and fractions containing FliZ protein were identified using Bradford solution and by SDS-page with subsequent coomasie staining. All purification steps were performed at 4°C and/or on ice.

3.4.20 Determination of protein concentrations

The absorbance of purified proteins was measured at 280 nm using the NanoDrop spectrophotometer and the concentration was calculated considering the size of the protein and its sequence-specific extinction coefficient.

3.4.21 Antibody production and purification

The polyclonal antibody against FliZ was produced by Pineda-Antikörper-Service (Berlin) in rabbits, using FliZ protein purified under denaturing conditions (3.4.19.1).

In order to remove antibodies that recognize *E. coli* proteins other than FliZ, the anti-FliZ serum was purified using an acetone powder prepared of the *fliZ* mutant as described by Harlow and Lane (Harlow and Lane 1999). To this end, 40 ml of an overnight-culture of the W3110 Δ*fliZ* mutant CP98 were harvested by centrifugation, cells were resuspended in 1 ml of 0.9 % NaCl and incubated on ice for 5 minutes. 4 ml of ice-cold acetone were added, the cell suspension was vortexed and incubated on ice for 30 minutes with occasional vortexing. After centrifugation, the precipitate was resuspended in fresh acetone, vortexed, incubated on ice for 10 minutes and after centrifugation, the precipitate was transferred to a filter paper and air-dried at room temperature. The resulting acetone powder was mixed with the anti-FliZ serum at a final concentration of approximately 1 % and incubated at 4°C for 30 minutes. The powder and antibodies bound to proteins therein were removed by centrifugation.

3.4.22 Protein-DNA binding analyses

3.4.22.1 Electrophoretic mobility shift assay (EMSA)

DNA fragments for the electrophoretic mobility shift assays were generated by PCR using the primers listed in table 3.6 at the end of this chapter. If plasmids carrying promoter DNA

fragments of the respective genes were available (see table 3.5) they were used as templates for the PCRs, otherwise extracted W3110 wild-type DNA was used as template DNA. The DNA fragments were purified by gel electrophoresis using 1.5 % agarose gels with subsequent gel extraction. Electrophoretic mobility shift assays were performed in bandshift buffer (10 mM Tris (pH 7.5), 1 mM EDTA, 5 % glycerol, 10 mM NaCl and 1 mM $MgCl_2$) in 20 µl reaction mixtures including increasing amounts (20-320 nM) of purified FliZ protein or FliZ-R108A mutant protein, 6 nM of DNA and 1 µg poly[d(I-C)] (Roche) as non-specific competitor DNA that blocks non-specific DNA binding by FliZ. Reaction mixtures were incubated for 20 min at room temperature and subsequently loaded onto native 5 % polyacrylamide gels (0.5 ml 10 x TBE Buffer, 1.25 ml of Rotiphorese Gel 40, 8.25 ml water, 16 µl TEMED, 66 µl APS). Gels were run in cold 0.5 x TBE buffer at 80 V for 100-120 minutes, stained with ethidium bromide and DNA bands were visualized by illumination with UV light.

3.4.22.2 Non-radioactive DNaseI footprint analysis

DNaseI footprint assays were performed as described before (Heroven et al. 2004; Mika and Hengge 2005) with minor alterations.

Preparation of DNA fragments for DNaseI footprint analysis

DIG-labelled DNA fragments for DNaseI footprint analysis were generated by PCR using the primers listed in table 3.6 at the end of this chapter and plasmids carrying promoter DNA fragments of the respective genes (see table 3.5) as template DNA. One of the two primers carried a DIG-label at the 5`end, thus labelling either the coding or the non-coding DNA strand. The DNA fragments were purified by gel electrophoresis using 1.5 % agarose gels with subsequent gel extraction.

DNA-protein complex formation

Complex formation between DIG-labelled DNA fragments (13 or 26 nM) and increasing amounts of FliZ (0.16-7.45 µM) was performed in 20 µl reaction mixtures as described above for the EMSA.

For MlrA-footprints, C-terminally 6xHis-tagged MlrA protein was provided by Sandra Lindenberg. The protein had been purified by a standard protocol for purification of 6xHis-tagged proteins under native conditions (Qiagen), using Tris (pH8) instead of NaH_2PO_4 in purification buffers (Sandra Lindenberg and Regine Hengge, unpublished data). For complex

formation between 13 nM of DIG-labelled DNA fragments and increasing amounts of MlrA (0-3.65 µM), 5 mM DTT was included into the bandshift buffer and poly[d(I-C)] was omitted. MlrA-DNA complex formation was performed at 28°C for 1 hour.

DNaseI digest

After DNA-protein complex formation, 5 µl of DNaseI (1u/µl, Fermentas) was added. The DNaseI digest was stopped after 10 seconds by addition of 50 µl of stop solution (15 mM EDTA, 10 µg/ml ytRNA in water). The amount of DNaseI and the time allowed for the DNaseI digest were occasionally increased if DNaseI activity had declined.

Phenol-chloroform extraction of DNA fragments

The DNA fragments were purified by phenol-chloroform extraction. 50 µl of PCI (phenol:chloroform:isoamyl alcohol, 25:24:1, pH 7.5-8.0) were added to each sample and carefully mixed with the sample by inverting the tube several times. Samples were centrifuged at maximal speed for 10 minutes at 20°C and the supernatant was mixed with 25 µl of 10 M ammonium acetate. The DNA was precipitated by addition of 300 µl of ice-cold 100 % ethanol and incubation for at least 30 minutes at -20°C. After centrifugation for 20 minutes at maximal speed the supernatant was removed and the pelleted DNA was washed with 70 % ethanol. The DNA pellet was air-dried at room-temperature, resuspended in 8 µl of footprint-sample buffer (10 mM EDTA (pH 8), 0.125 % xylene cyanole, 0.125 % bromphenol blue, in formamide) and samples were stored at -20°C until they were run on denaturing sequencing gels.

Generation of DIG-labelled sequencing reactions

In order to be able to identify the sequences protected from DNaseI digestion by FliZ/MlrA binding, a DNA sequence ladder was generated with the same DIG-labelled primer used for amplification of the footprint DNA fragment. The sequencing reactions were generated with the CycleReader DNA Sequencing Kit (Fermentas) according to the manufacturer´s protocol, using 1.5 pmol of the labelled primer and 100 fmol of template DNA, which had been generated by PCR, using unlabeled primers identical to the ones used for amplification of the footprint DNA fragment.

Separation of DNA fragments on denaturing sequencing gels

The DNA fragments generated in the DNaseI digests were separated on denaturing 6 % polyacrylamide sequencing gels containing 7 M urea (42 g urea, 10 ml 10x TBE, 15 ml Rotiphorese Gel40, 50 µl TEMED, 500 µl 10 % APS in a total volume of 100 ml) using a gel electrophoresis system from C.B.S. Scientific CO. One of the glass plates between which the gel was poured had been silanized with Sigmacote (Sigma-Aldrich) before pouring to ensure that it can be easily removed from the gel later. The gel was allowed to polymerize for at least two hours or overnight. Before the samples were loaded, the gel was pre-warmed by a 30 minutes pre-run at 50 W. The DNaseI footprint samples and approximately 0.5-1.5 µl of the sequencing reactions, which were added up to a total volume of 8 µl with footprint-sample buffer, were heated to 85°C prior to loading. The run was started at 50 W and continued at 40 W after the samples had entered the gel matrix. Gels were run for approximately 100 minutes until the bromphenol blue contained in the footprint-sample buffer had run out of the gel.

Southern Blot

The separated DNA fragments were transferred to a NYTRAN N nylon membrane (0.2 µm pore size, Whatman) by southern blot. To this end, the silanized glass plate was removed from the gel and the membrane was put on the gel and covered with 5 layers of blotting paper (Whatman, 3MM) and a glass plate. After 2 hours, the membrane was removed from the gel and the DNA was UV-cross-linked to the membrane for 4 minutes.

Detection of DIG-labelled DNA

The DIG-labelled DNA-fragments were visualized by an alkaline phosphatase-catalyzed chemiluminescence reaction using the ultra-sensitive substrate CDP-Star (Roche).

To this end, the membrane was washed in 100 ml of washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3 % Tween 20, pH 7.5) for 1-5 minutes and blocked in 100 ml of blocking solution (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 1 % blocking reagent (Roche)) for 30 minutes before 10 µl of an anti-digoxigenin-alkaline phosphatase antibody (Roche) (1:10 000 dilution) were added. After incubation with shaking at room temperature for 1 hour, excess antibody was removed by washing the membrane 4 times with 100 ml of washing buffer for 10 minutes. The membrane was then equilibrated for 5 min in detection buffer (0.1 M Tris (pH 9.5), 0.1 M NaCl, pH 9.5) before it was covered with 15 ml of CDP-Star in detection buffer (11 ml detection buffer, 4ml CDP-Star solution) in the dark. After 5 min of incubation, the CDP-Star solution was removed, the membrane was covered with foil and the

chemiluminescent signal was detected with a chemiluminescent detection film (Thermo Scientific), which was exposed to the membrane for 10-30 minutes.

3.4.23 Limited proteolysis experiments

The digestion pattern and kinetics of purified proteins that are subjected to limited proteolysis by proteinases like trypsin and proteinase K change with the folding state of the proteins, since protein parts that show local unfolding provide better access to the enzymes for cleavage (Fontana et al. 2004). Therefore, limited proteolysis can be used to compare the overall stabilities of identical or nearly identical proteins *in vitro*. To compare the overall stabilities of the purified wild-type FliZ protein and the FliZ-R108A mutant, the proteins were incubated with 0.2 BAEE units (12.8 ng) of TPCK-Trypsin (Thermo-Scientific; dissolved in water) per mg of protein or with 1.875 ng of proteinase K (Sigma; dissolved in 50 mM Tris (pH 8), 10 mM $CaCl_2$) per mg of protein in FliZ purification buffer D (see 3.4.19.2) at 25°C for increasing time intervals. Proteolysis reactions were stopped by the addition of SDS-PAGE sample buffer and digestion products were analyzed by SDS-page with subsequent coomassie staining.

3.4.24 *In vivo* protein-protein interaction analysis: Bacterial two-hybrid assay

The bacterial two-hybrid system is an efficient method to detect protein-protein interactions *in vivo*. In this work, the BacterioMatch II Two-Hybrid System from Agilent Technologies was used. In order to test whether two proteins interact with each other, one protein (bait protein) is expressed from vector pBT as a protein fusion to the full-length bacteriophage λ repressor, while the potential interaction partner (target protein) is expressed from vector pTRG as a protein fusion to the N-terminal domain of the α subunit of RNA polymerase. If the two proteins interact, they bind together to the λ operator site upstream of the HIS3 reporter gene. This leads to recruitment of RNAP to the promoter and stabilizes RNAP binding, thereby activating transcription of the HIS3 gene, which complements the histidine auxotrophy of the reporter strain. If induction of HIS3 expression in the presence of bait-target interaction is strong enough, it allows for growth in the presence of 2-amino-1,2,4-triazole (3-AT), which is a competitive inhibitor of the His3 enzyme.

The pBT-RpoD and pTRG-FliZ plasmids were constructed using the primers listed in table 3.6 at the end of this chapter. The pBT-RpoS and pTRG-RssB plasmids were obtained from Eberhard Klauck (Eberhard Klauck and Regine Hengge, unpublished data).
The bacterial two-hybrid analyses were basically performed according to the manufacturer´s instruction manual with the following alterations:

The selective screening medium was prepared with a final concentration of 3.5 mM 3-AT. For co-transformations of reporter cells, 50 µl aliquots of competent reporter cells were transformed with 1.5 µl of the respective plasmids and the amounts of media and chemicals used in the co-transformation protocol were adapted to this aliquot size. However, the co-transformed cells were incubated in 1 ml of $M9^+$ His-dropout medium (prepared according to the instructions manual) for 2 h before plating. After co-transformantions with one or both of the empty plasmids (pBT or pTRG), 400 µl of a 1:100 dilution (diluted in $M9^+$ His-dropout medium) of the co-transformation mixture were plated on non-selective screening medium and 400 µl of the undiluted co-transformation mixture were plated on selective screening medium. After co-transformantions with combinations of pBT- and pTRG-derivatives carrying the *fliZ*, *rpoD*, *rpoS* and *fliZ* genes, 200 µl of the undiluted co-transformation mixture were plated on both selective and non-selective screening medium.

Growth of the co-transformants on selective and non-selective medium was compared after overnight incubation at 37°C and 20-50 co-transformants of each vector combination were patched from non-selective medium onto large plates containing selective or non-selective medium and incubated overnight at 37°C, or for 3-4 days at 28°C. The additional patching step was introduced as growth of cells co-transformed with different vector combinations on one plate allowed for a better and more direct comparison of these different transformants.

3.5 Databases and bioinformatic analyses

3.5.1 Gene sequences and annotations

Gene sequences and annotations were obtained from databases on the following websites:

- NCBI (*www.ncbi.nlm.nih.gov/*)
- EcoCyc (*ecocyc.org*)
- coliBase (*www.xbase.ac.uk/colibase/*)

3.5.2 Protein and DNA sequence alignments

For multiple-sequence alignments of DNA and protein sequences, the ClustalW2 program (Chenna et al. 2003) on the website of the European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI) (*www.ebi.ac.uk/Tools/msa/clustalw2*) was used.

For the alignment of the C-terminal part of FliZ with the core-binding domain of XerD, Cre and λ phage integrase, a conserved domain search using the NCBI CD-search interface (Marchler-Bauer and Bryant 2004) (*www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi*) was performed and based on the obtained results, FliZ was added to the alignment in Swalla et al. (Swalla et al. 2003).

3.6 Bacterial strains, bacteriophages, plasmids and oligonucleotide primers used in this work

3.6.1 Bacterial strains

Tab. 3.3: Bacterial strains used in this work. For strains with *lacZ* fusions the end points of the fused promoter/gene fragments are noted with numbers indicating the position relative to the translational start site of the respective gene.

Wild-type strains		
Strain	**Genotype**	**Reference**
MC4100	*E.coli* K12 F- *araD*139 Δ(*argF-lac*)U169 *deoC flbB*5301 *relA*1 *rpsL*150 *ptsF*25 *rbsR*	(Silhavy et al. 1984) (Peters et al. 2003)
W3110	*E.coli* K12 *thyA36 deoC2 IN(rrnD-rrnE)I*	(Hayashi et al. 2006)
W3110 Δ*lac*U169	W3110Δ(*argF-lacU*)169 *zaj-3053*::Tn*10*	(Nichols et al. 1998) (Peters et al. 2003)
Derivatives constructed by other members of the Hengge group		
Strain	**Genotype**	**Reference**
AK68	NS125 *fliZ::kan*	(Pesavento et al. 2008)
AK74	NS125 Δ*fliA*	(Pesavento et al. 2008)
AK75	NS125 *crl::cat*	(Pesavento et al. 2008)
AK78	NS125 *rsd::cat*	(Pesavento et al. 2008)
AM125	MC4100 *clpP::cat*	(Muffler et al. 1997)
AP33	W3110 Δ*lac*U169 [λRS45:*rtn(-298, +52)::lacZ* (hybr)]	(Sommerfeldt et al. 2009)
AP34	W3110 Δ*lac*U169 [λRS45:*yhjH(-279, +49)::lacZ* (hybr)]	(Sommerfeldt et al. 2009)
AP36	W3110 Δ*lac*U169 [λRS45:*yjcC(-287, +43)::lacZ* (hybr)]	(Sommerfeldt et al. 2009)
AP37	W3110 Δ*lac*U169 [λRS45:*ylaB(-300, +31)::lacZ* (hybr)]	(Sommerfeldt et al. 2009)
AP60	W3110 Δ*lac*U169 [λRS74:*yoaD(-273; +58)::lacZ*]	(Sommerfeldt et al. 2009)
AP69	W3110 Δ*lac*U169 *rtn::kan*	(Sommerfeldt et al. 2009)
AP70	W3110 Δ*lac*U169 *yjcC::kan*	(Sommerfeldt et al. 2009)
AP87	MC4100 *ylaB::kan*	(Sommerfeldt et al. 2009)
AP135	AP34 *clpP::cat*	(Pesavento et al. 2008)
CAB11	MC4100 *fliA::cat*	(Barembruch 2007)
CAB85	W3110 Δ*lac*U169 [λRS74:*flgA(-121; +17)::lacZ*]	Claudia Barembruch and Regine Hengge, unpublished data
FS20	MC4100 *rpoS::kan*	(Mika and Hengge 2005)
GB206	AP34 Δ*fliZ*	Giesela Klauck and Regine Hengge, unpublished data
GB332	W3110 *flhDC::kan*	(Pesavento et al. 2008)
GB350	W3110 Δ*lac*U169 [λRS45:*csgB(-190, +43)::lacZ* (hybr)]	(Pesavento et al. 2008)
GB391	GB350 Δ*yhjH*	(Pesavento et al. 2008)
GB812	GB350 Δ*fliZ yhjH::cat*	Gisela Klauck and Regine Hengge; unpublished data
HW103	MC4100 *ydaM::cat*	(Weber et al. 2006)
HW104	MC4100 *yciR::kan*	(Weber et al. 2006)
HW107	MC4100 *yddV::*Tn*5*	(Weber et al. 2006)
HW125	MC4100 *yddU::cat*	(Weber 2007)
HW129	MC4100 *mlrA(-199, +91)::lacZ*	(Weber et al. 2006)
HW142	MC4100 [λRS45 *csgB(-190, +43)::lacZ* (hybr)]	(Weber et al. 2006)
HW152	HW142 *yciR::kan*	(Weber et al. 2006)

Tab. 3.3 (continued)

Strain	Genotype	Reference
HW154	HW142 *ydaM::cat*	(Weber et al. 2006)
HW159	MC4100 *ycdT::cat*	(Weber 2007)
JK79	MC4100 [λRS45:*gadB*(-231; +138)::*lacZ* (hybr)]	Johanna Heuveling and Regine Hengge, unpublished data
MB30	MC4100 *hns205::*Tn*10*	Mechthild Barth, strain collection AG Hengge
NS20	MC4100 *yeaJ::kan*	Nicole Sommerfeldt, and Regine Hengge, unpublished data
NS23	HW142 *yeaJ::kan*	Nicole Sommerfeldt, and Regine Hengge, unpublished data
NS34	W3110 *yoaD::kan*	(Sommerfeldt et al. 2009)
NS49	W3110 *yciR::kan*	Nicole Sommerfeldt, and Regine Hengge, unpublished data
NS123	W3110 Δ*lac*U169 [λRS45:*ydaM(-307, +43)::lacZ* (hybr)]	(Pesavento et al. 2008)
NS125	W3110 Δ*lac*U169 [λRS45:*mlrA(-199, +91)::lacZ* (hybr)]	(Pesavento et al. 2008)
NS127	W3110 Δ*lac*U169 [λRS45:*yddV(-391, +49)::lacZ* (hybr)]	(Sommerfeldt et al. 2009)
NS149	W3110 Δ*lac*U169 [λRS45:*yciR(-296, +64)::lacZ* (hybr)]	(Sommerfeldt et al. 2009)
RO151a	MC4100 f(*osmY*(*csi*-5::*lacZ*)(λplacMu55)	(Lange and Hengge-Aronis 1991)

Derivatives constructed in this work/in earlier studies

Strain	Genotype	Reference
CP2	MC4100 [λRS45:*ydaM(-307, +43)::lacZ* (hybr)]	(Weber et al. 2006)
CP5	MC4100 [λRS45:*yciR(-296, +64)::lacZ* (hybr)]	(Weber et al. 2006)
CP13	MC4100 *yedQ::cat*	(Weber et al. 2006)
CP30	HW142 *yddV::*Tn*5*	(Weber et al. 2006)
CP47	CP47 MC4100 *yaiC::kan*	(Weber et al. 2006)
CP50	CP5 *hns205::*Tn*10*	this work
CP57	CP2 *hns205::*Tn*10*	this work
CP58	HW142 *yaiC::kan*	this work
CP60	HW152 *ydaM::cat*	this work
CP63	HW152 *yedQ::cat*	this work
CP64	HW142 Δ*yciR*	this work
CP66	CP64 *yaiC::kan*	this work
CP67	CP64 *yddV::*Tn*5*	this work
CP68	CP64 *ycdT::cat*	this work
CP69	HW142 *ycdT::cat*	this work
CP70	HW152 *ycdT::cat*	this work
CP72	HW142 *fliA::cat*	this work
CP76	CP64 *yeaJ::kan*	this work
CP87	HW142 Δ*fliA*	this work
CP91	MC4100 *fliZ::kan* (pKD4)	this work
CP92	HW142 *fliZ::kan*	this work
CP96	HW142 Δ*fliZ*	this work
CP97	GB350 *fliZ::kan*	this work
CP98	GB350 Δ*fliZ*	this work

Tab. 3.3 (continued)

Strain	**Genotype**	**Reference**
CP99	GB350 *clpP::cat*	this work
CP100	W3110 *fliZ::kan* (pKD4)	this work
CP105	MC4100 *ygeH::kan* (pKD4)	this work
CP106	HW142 *ygeH::kan*	this work
CP107	CP99 *fliZ::kan*	this work
CP108	CP99 Δ*fliZ*	this work
CP109	CAB85 *fliZ::kan*	this work
CP112	CAB85 Δ*fliZ*	this work
CP115	GB391 *clpP::cat*	this work
CP119	NS123 *fliZ::kan*	this work
CP124	NS123 Δ*fliZ*	this work
CP128	CP99 *flhDC::kan*	this work
CP132	GB350 *flhDC::kan*	this work
CP133	W3110 Δ*lac*U169 [λRS45:*fliAZ(fliA-237, fliZ+86)::lacZ*]	this work
CP134	CP133 *rpoS::kan*	this work
CP136	CP133 *fliZ::kan*	this work
CP150	NS149 *fliZ::kan*	this work
CP154	NS149 Δ*fliZ*	this work
CP158	NS127 *fliZ::kan*	this work
CP159	AP36 *fliZ::kan*	this work
CP160	AP37 *fliZ::kan*	this work
CP161	CP99 *fliA::kan*	this work
CP162	NS127 Δ*fliZ*	this work
CP163	AP36 Δ*fliZ*	this work
CP164	AP37 Δ*fliZ*	this work
CP165	CP99 Δ*fliA*	this work
CP166	GB812 *rtn::kan*	this work
CP167	GB350 Δ*fliZ* Δ*yhjH*	this work
CP168	GB812 *yoaD::kan*	this work
CP169	GB812 *yciR::kan*	this work
CP170	GB812 *yjcC::kan*	this work
CP171	GB812 *ylaB::kan*	this work
CP172	CP167 *yddU::cat*	this work
CP173	AP33 *fliZ::kan*	this work
CP174	AP60 *fliZ::kan*	this work
CP175	AP33 Δ*fliZ*	this work
CP176	AP60 Δ*fliZ*	this work
CP188	NS125 Δ*fliZ*	this work
CP192	GB350 Δ*flhDC*	this work
CP193	GB350 Δ*flhDC clpP::cat*	this work
CP195	CP192 *rpoS::kan*	this work
CP196	CP193 *rpoS::kan*	this work
CP197	W3110 *fliY::kan* (pKD13)	this work
CP198	GB350 *fliY::kan*	this work
CP201	W3110 *nsrR::kan* (pKD4)	this work
CP209	CP133 *nsrR::kan*	this work
CP217	CP133 Δ*nsrR*	this work
CP219	W3110 Δ*lac*U169 [λRS45:*nsrR(-210, +40)::lacZ* (hybr)]	this work
CP222	CP219 *rpoS::kan*	this work
CP223	CP219 *fliZ::kan*	this work
CP225	CP219 Δ*fliZ*	this work
CP230	W3110 Δ*lac*U169 [λRS74:*gadE(-91, +31)::lacZ* (hybr)]	this work

Tab. 3.3 (continued)		
Strain	**Genotype**	**Reference**
CP232	CP230 *rpoS::kan*	this work
CP233	CP230 *fliZ::kan*	this work
CP236	CP230 Δ*fliZ*	this work
CP250	W3110 Δ*lac*U169 [λRS74:*flhDC1(flhD-1594,+35)::lacZ*]	this work
CP256	CP250 *rpoS::kan*	this work
CP257	CP250 *fliZ::kan*	this work
CP262	W3110 Δ*lac*U169 [λRS45:*flhDC2-new(flhD-1594, -180)::lacZ*]	this work
CP264	W3110 Δ*lac*U169 [λRS74:*flhDC3-new(flhD-215, +35)::lacZ*]	this work
CP267	CP262 *fliZ::kan*	this work
CP271	CP264 *fliZ::kan*	this work
CP274	CP262 Δ*fliZ*	this work
CP276	CP264 Δ*fliZ*	this work
CP278	CP250 Δ*fliZ*	this work
Strains used for FliZ over-expression		
ER2566	F– λ– fhuA2 [lon] ompT lacZ::T7 gene1 gal sulA11 Δ(mcrC-mrr)114::IS10 R(mcr-73::miniTn10–TetS)2 R(zgb-210::Tn10)(TetS) endA1 [dcm]	New England Biolabs
FI1202	*lacI*$^{q+}$ *lacL8 glnG*::Tn5 λ202	(Fiedler and Weiss 1995)

3.6.2 Bacteriophages

Tab. 3.4: Bacteriophages used in this work.

Lysat	**Reference**
λRS45	(Simons et al. 1987)
λRS74	(Simons et al. 1987)
$P1_{vir}$	Laboratory collection Hengge group

3.6.3 Plasmids

Tab. 3.5: Plasmids used in this work. For pJL28 and pCAB6 derivatives with *lacZ* fusions the end points of the fused promoter/gene fragments are noted with numbers indicating the position relative to the translational start site of the respective gene. Antibiotic resistance encoded by the respective plasmid is noted as follows: AmpR: ampicillin resistance, KanR: kanamycin resistance, CmR: chloramphenicol resistance, TetR: tetracylin resistance

Cloning vectors and plasmids generated elsewhere		
Plasmid	**Description**	**Reference**
pAP6	pJL28 derivative with *ylaB(-300, +31)::lacZ*	(Sommerfeldt et al. 2009)
pAB17	pJL28 derivative with *yjcC(-287, +43)::lacZ*	(Sommerfeldt et al. 2009)
pAP22	pJL28 derivative with *flhDC(flhC-1956,+46)::lacZ*	(Sommerfeldt et al. 2009)
pBAD18	Arabinose-inducible vector carrying the promoter of the *araBAD* operon and *araC*, AmpR	(Guzman et al. 1995)
pBAD18-YdaM	pBAD18 derivative with the *ydaM* gene	(Weber et al. 2006)
pBT	BacterioMatch II Two-Hybrid System bait vector; encodes the full-length bacteriophage λ repressor, CmR	Agilent Technologies

(Tab. 3.5 (continued)

Plasmid	Description	Reference
pBT-RpoS	pBT-derivative expressing RpoS fused to the full-length bacteriophage λ repressor	Eberhard Klauck and Regine Hengge, unpublished data
pCAB6	vector for the generation of transcriptional *lacZ* fusions, AmpR	(Barembruch and Hengge 2007)
pCAB7	pCAB6 derivative with *flgA(-121; +17)::lacZ*	(Barembruch and Hengge 2007)
pCAB8	pCAB6 derivative with *flgM(-133, +35::lacZ*	(Barembruch and Hengge 2007)
pCAB18	IPTG-inducible low copy number vector carrying the p_{tac} promoter, AmpR	(Barembruch and Hengge 2007)
pCAB19	pCAB18 derivative with the *flhDC* operon	(Barembruch and Hengge 2007)
pCP20	helper plasmid for eliminating antibiotic resistance genes after one-step inactivation; encodes FLP recombinase, synthesis of FLP is temperature-inducible; temperature-sensitive replication; AmpR, CmR	(Datsenko and Wanner 2000)
pFS1	pRL45 derivative with a *rpoS742::lacZ* fusion	(Mika and Hengge 2005)
pHW2	pJL28 derivative with *gadE(-463,+60)::lacZ*	(Weber et al. 2005)
pHW4	pQE60 derivative with the *mlrA* coding region	(Weber 2007)
pHW5	pJL28 derivative with *mlrA(-199, +91)::lacZ*	(Weber et al. 2006)
pHW7	pJL28 derivative with *csgD(-756,+85)::lacZ*	(Weber 2007)
pJL28	vector for the generation of *lacZ* fusions, AmpR	(Lucht et al. 1994)
pJV300	high copy number control vector expressing a nonsense RNA under the control of the p_{LlacO} promoter	(Urban and Vogel 2007)
pKD4	Template plasmid for one-step inactivation, carrying the *kan*-cassette; AmpR, KanR	(Datsenko and Wanner 2000)
pKD13	Template plasmid for one-step inactivation, carrying the *kan*-cassette; AmpR, KanR	(Datsenko and Wanner 2000)
pKD46	helper plasmid for one-step inactivation; encodes the λ Red recombinase; temperature-sensitive replication; AmpR	(Datsenko and Wanner 2000)
pNAT58	pCAB18 derivative expressing a YmgB-Strep fusion protein	Natalia Tschowri and Regine Hengge, unpublished data
pRH800	IPTG-inducible vector carrying the p_{tac} promoter, AmpR	(Lange and Hengge-Aronis 1994)
pRep4	Helper plasmid for protein over-expression, that constitutively expresses the *lac* repressor at high levels, KanR	Qiagen
pTRG	BacterioMatch II Two-Hybrid System target vector; encodes the RNAP αNTD, TetR	Agilent Technologies
pTRG-RssB	pTRG-derivative expressing RssB fused to the RNAP αNTD	Eberhard Klauck and Regine Hengge, unpublished data
pTYB12	Protein expression vector of the IMPACT-CN system for expression of fusion proteins with a N-terminal self-cleavable intein tag, AmpR	New England Biolabs
pQE30-XA	Protein expression vector for expression of proteins with a N-terminal 6xHis-tag and a Xa protease recognition site for removal of the 6xHis-tag	Qiagen

Tab. 3.5 (continued)

Plasmid	Description	Reference
pZE12-*luc*	Protein expression vector carrying the luciferase gene under the control of the p_{LlacO} promoter, AmpR	(Lutz and Bujard 1997)

Constructs generated in this work/earlier studies

Plasmid	Description	Reference
pBAD-YdaM-EE334/335AA	pBAD derivative with a mutated *ydaM* gene encoding a YdaM mutant with the amino acid glutamate at positions 334 and 335 in the GGEEF motif exchanged by alanine (A)	this work
pBT-RpoD	pBT-derivative expressing RpoD fused to the full-length bacteriophage λ repressor	this work
pCAB6-*flhDC1*	pCAB6 derivative with *flhDC(-1594, +35)::lacZ*	this work
pCAB6-*flhDC2*-new	pCAB6 derivative with *flhDC2-new(-1594, -180)::lacZ*	this work
pCAB6-*flhDC3*-new	pCAB6 derivative with *flhDC3-new(-215, +35)::lacZ*	this work
pCAB6-*fliZ*	pCAB6 derivative with *fliAZ(fliA-237, fliZ+86)::lacZ*	this work
pCP2	pJL28 derivative with *ydaM(-307, +43)::lacZ*	(Weber et al. 2006)
pCP5	pJL28 derivative with *yciR(-296, +64)::lacZ*	(Weber et al. 2006)
pFliZ	pCAB18-derivative with the *fliZ* gene	this work
pFliZ-R108A	pCAB18 derivative with a mutated *fliZ* gene encoding a FliZ mutant with the amino acid arginine (R) at position 108 exchanged by alanine (A)	this work
pFliY	pCAB18 derivative with the *fliY* gene	this work
pJL28-*gadE*-short	pJL28 derivative with *gadE(-91, +31)::lacZ*	this work
pJL28-*nsrR*	pJL28 derivative with *nsrR(-210, +40)::lacZ*	this work
pJL28-*yciR*-C-23T	like pCP5, with a cytosine to thymine exchange at position -23 relative to the transcriptional start site	this work
p*mlrA*1-C-13G	like pHW5, with a cytosine to guanine exchange at position -13 relative to the transcriptional start site	this work
p*mlrA*2-TC-14/-13GG	like pHW5, with a thymine to guanine exchange at position -14 and a cytosine to guanine exchange at position -13 relative to the transcriptional start site	this work
p*mlrA*3-T-6C	like pHW5, with a thymine to cytosine exchange at position -6 relative to the transcriptional start site	this work
p*mlrA*5-T-7A	like pHW5, with a thymine to adenine exchange at position -6 relative to the transcriptional start site	this work
p*mlrA*6-T-12A	like pHW5, with a thymine to adenine exchange at position -12 relative to the transcriptional start site	this work
p*mlrA*7-AA-22/-21TT	like pHW5, with adenine to thymine exchanges at positions -22 and -21 relative to the transcriptional start site	this work
p*mlrA*8-C-24T	like pHW5, with a cytosine to thymine exchange at position -12 relative to the transcriptional start site	this work
pQE30-XA-FliZ	pQE30-XA derivative with the *fliZ* gene	this work
pRH800-YciR	pRH800 derivative with the *yciR* gene	this work
pRH800-YciR-DE316/317AA	pRH800 derivative with a mutated *yciR* gene encoding a YciR mutant with the amino acids aspartate (D) at position 316 and glutamate (E) at position 217 in the GGDEF motif exchanged by alanines (A)	this work

Tab. 3.5 (continued)		
Plasmid	**Description**	**Reference**
pRH800-YciR-E440A	pRH800 derivative with a mutated *yciR* gene encoding a YciR mutant with the amino acid glutamate (E) at position 440 in the EAL motif exchanged by alanine (A)	this work
pRyeB	high copy number control vector expressing the *ryeB* gene under the control of the p_{LlacO} promoter	this work
pTRG-FliZ	pTRG-derivative expressing FliZ fused to the RNAP αNTD	this work
pTYB12-FLiZ	pTYB12 derivative with the *fliZ* gene	this work
pTYB12-FliZ-R108A	pTYB12 derivative with a mutated *fliZ* gene encoding a FliZ mutant with the amino acid arginine (R) at position 108 exchanged by alanine (A)	this work

3.6.4 Primers

Tab. 3.6: Oligonucleotide primers used in this work. Restriction sites are indicated by bold italic letters, point mutations in primers used for recombinant PCR are indicated by bold letters. Phosporylated primers are indicated by "Pho" and "DIG" denotes a digoxigenin lable. Primers generated by other members of the Hengge group (unpublished data) or in other studies are indicated.

I. Primers used for generating *lacZ* fusions		
Primers for cloning gene fusion fragments into pJL28 or pCAB6		
lacZ fusion	*Primer name*	*Sequence*
flhDC1(-1594, +35)::lacZ	P*flhD-BamH*I P*flhD-Hind*III (both primers constructed by Alexandra Possling)	5′-CG***GGATCC***CATCCCATTTCGATTATTCC-3′ 5′-CCC***AAGCTT***GTTTCAGCAACTCGGAGG-3′
flhDC2-new(-1594, -180)::lacZ	P*flhD-BamH*I P*flhD-lacZ*-2-*Hind*III-new	see above 5′-AGC***AAGCTT***CCTAAATCGACGCAACTGT AC-3′
flhDC3-new(-215, +35)::lacZ	PflhD3-BamHI-new P*flhD-Hind*III	5′-CG***GGATCC***CGTGTAGTGACGAGTACAGTTGC-3′ see above
fliAZ(fliA-237, fliZ+86)::lacZ	P*fliA*5-*BamH*I (constructed by Claudia Barembruch) P*fliZ-lacZ-Hind*III	5′-GTT***GGATCC***CAATTTATTGAATTTGCAC-3′ 5′-CAGC***AAGCTT***CGGCAATGCGCGCAATGGG TCTG-3′
gadE(-91, +31)::lacZ	P*gadE-lacZ*-short-*Eco*RI P*gadE-lacZ-Hind*III	5′-GCG***AATTCGG***CGTTTACTATATTGAACAA CG-3′ 5′-CGTG***AAGCTT***GAAAAGAATCTTTCGTCAT GAG-3′
nsrR(-210, +40)::lacZ	P*nsrR-lacZ-Eco*RI P*nsrR-lacZ-Hind*III	5′-GC***GAATTC***GCGTAATTCTGGTACGCCTGG CAG-3′ 5′-GCCG***AAGCTT***GCGCACGTAATCCGTAATC AG-3′
Primers for testing single lysogeny of chromosomal *lacZ* fusions (Powell et al. 1994)		
Primer name	*Sequence*	
P1	5′-GAGGTACCAGCGCGGTTTGATC-3′	
P2	5′-TTTAATATATTGATATTTATATCATTTTACGTTTCTCGTTC-3′	
P3	5′-ACTCGTCGCGAACCGCMC-3′	

Tab. 3.6 (continued)

II. Primers used for generating knockout mutations

Primers for testing knockout mutations constructed by one step inactivation (Datsenko and Wanner 2000)

Primer name	*Sequence*
k1	5′-CAGTCATAGCCGAATAGCCT-3′
k2	5′-CGGTGCCCTGAATGAACTGC-3′
c1	5′-TTATACGCAAGGCGACAAGG-3′
c2	5′-GATCTTCCGTCACAGGTAGG-3′

Gene-specific primers used for constructing and testing knockout mutations

Mutation	*Primer name*	*Sequence*
fliZ::kan	M*fliZ*-P1	5′-TAAATGCCGCACTTTAACTTTGACTACCAGGAGTT CTTAATG ATGGTGCAGTGTAGGCTGGAGCTGCTTC-3′
	M*fliZ*-P2	5′-CCATTGTTTGTAAACACAAAAACAACTCCGCTAC ATCTTATT CTTATTTACATATGAATATCCTCCTTAG-3′
	M*fliZ*-Control-P1	5′-GCGGGTCAGTCAGTTACACAGCCAGGC-3′
	M*fliZ*-Control-P2	5′-CCATCAATGCCTGACGTCCCAGATGTGC-3′
fliY::kan	M*fliY*-P1	5′- CAAACAATGGCTCTACACTGCAAACAGACATAAC AACATTCGGGGTGAATGTGTAGGCTGGAGCTGCTTC-3′
	M*fliY*-P4	5′- GTAAATAAAAAAGGCGCTAGTGAAAGCGCCCTTTTT TGTCATTATGCTGAATTCCGGGGATCCGTCGACC-3′
	M*fliY*-Control-P1	5′-CCTCCAGCCTGCCTTCTTCTG-3′
	M*fliY*-Control-P2	5′-ATTCGAGCGGCGTTGGCGCGCCG-3′
nsrR::kan	M*nsrR*-P1	5′-TTATCATCAATATAAATGTATTTTTTCCCGATTTCCCTT TTGAG GTTGATGTGTAGGCTGGAGCTGCTTC-3′
	M*nsrR*-P2	5′-GGATTCGCGTATTTTTCAGCTTCGCGTTCCTGGAAAG GATCT TGTGACATATGAATATCCTCCTTAG-3′
	M*nsrR*-Control-P1	5′-GCCATTACGCTATCCGACAC-3′
	M*nsrR*-Control-P2	5′-ACCAGTTGACCATCGCGCTC-3′
ygeH::kan	M*ygeH*-P1	5′- GCAGGATGCAAGAAACCAATTTTTTCATAGAGGTTA ACTAAT GGACTTAGGTGTAGGCTGGAGCTGCTTC-3′
	M*ygeH*-P2	5′- GTTAGGCACATACATATCTACACATTCTTTTATCACAA CTGCT TTTCGTCCATATGAATATCCTCCTTAG-3′
	M*ygeH*-Control-P1	5′-CCACTATAACCAGCACCTC-3′
	M*ygeH*-Control-P2	5′-CTCGATGGCTTTCACTCTC-3′

III. Primers for cloning and mutagenizing *fliZ* into pCAB18

Outside/cloning primers

Primer name	*Sequence*
P*fliZ*-pRH800-*Eco*RI	5′-GC***GAATTC***GCCGCACTTTAACTTTGACTACCAGGAG-3′
P*fliZ*-pRH800-*Hind*III	5′-CGTGCG***AAGCTT***TTAATATATATCAGAAGAAGGCAGGCTGGA GG-3′

Inside mutagenesis primers

Primer name	*Sequence*
P*fliZ*-R108A-f	5′-CCCAGGTACGGTG**GC**TGAATATGTCGTTCG-3′
P*fliZ*-R108A-r	5′-CGAACGACATATTCA**GC**CACCGTACCTGGG-3′

IV. Primers for cloning *fliZ* and *fliZ(R108A)* into pTYB12

Primer name	*Sequence*
P*fliZ*-pTYB1/12-for(*Nde*I)	5′-GGTGGT***CATATG***ATGGTGCAGCACCTG-3′
P*fliZ*-pTYB12-rev(*Eco*RI)	5′-TGGT***GAATTC***TCAATATATATCAGAAGAAGGCAGGCTG GAGG-3′

Tab. 3.6 (continued)

V. Primers for cloning *fliZ* into pQE30-XA

Primer name	*Sequence*
P*fliZ*-pQE30-XA	5′-Pho-ATGATGGTGCAGCACCTGAAAAGACG-3′
P*fliZ*-pRH800-*Hind*III	see above

VI. Primers for cloning *fliY* into pCAB18

Primer name	*Sequence*
P*fliY*-pCAB18-for	5′-CG***GAATTC***AGGAGGGTGAATATGAAATTAGCACATCTGG-3′
P*fliY*-pCAB18-rev	5′-CGTGCG***AAGCTT***TTATTTGGTCACATCAGCACC-3′

VII. Primers used for constructing pRyeB

Primers for amplifying *ryeB*

Primer name	*Sequence*
PryeB-for-Pho	5′-Pho-GCAAGGCAACTAAGCCTGC-3′
PryeB-rev (*Xba*I)	5′-GC***TCTAGA***AAAAGAGACCGAACACG-3′

Primers for amplifying the vector backbone of pZE12-*luc* (Urban and Vogel 2007)

Primer name	*Sequence*
PLlacOB	5′-CGCACTGACCGAATTCATTAA-3′
PLlacOC	5′-GTGCTCAGTATCTTGTTATCCG-3′

VIII. Primers for cloning *fliZ* and *rpoD* into plasmids of the bacterial two-hybrid system

Construct	*Primer name*	*Sequence*
pTRG-FliZ	BTH-FliZ-*Eco*RI	5′-GC***GAATTC***AGATGATGGTGCAGCACCTG-3′
	BTH-FliZ-*Xho*I	5′-CG***CTCGAG***TTAATATATATCAGAAGAAGGCAGGCTGGAGG-3′
pBT-RpoD	BTH-RpoD-*Eco*RI	5′-GC***GAATTC***CATGGAGCAAAACCCGCAGTCACAGC-3′
	BTH-RpoD-*Xho*I	5′-CG***CTCGAG***TTAATCGTCCAGGAAGCTACGCAGCAC -3′

IX. Primers for cloning and mutagenizing *yciR* on pRH800

Outside/cloning primers

Primer name	*Sequence*
P*yciR*-*Eco*RI	5′-GC***GAATTC***GCTGTTAACCGGAGGATATGC-3′
P*yciR*-*Hind*III	5′-CGTGAC***AAGCTT***TTATGCGCGCTTCAGATAGCG-3′

Inside mutagenesis primers

Mutation	*Primer name*	*Sequence*
YciR-DE316/317AA	P*yciR*-GGAAF-f	5′-CGTCCAGGTGGGG**C**TG**C**GTTTCTGGTACTGGC-3′
	P*yciR*-GGAAF-r	5′-GCCAGTACCAGAAAC**G**CA**G**CCCCACCTGGACG-3′
YciR-E440A	P*yciR*-AAL-f	5′-CGCAGTCTGG**C**AGCACTAGTACG-3′
	P*yciR*-AAL-r	5′-CGTACTAGTGCT**G**CCAGACTGCG-3′

X. Primers for cloning and mutagenizing *ydaM* on pBAD18

Outside/cloning primers (Weber et al. 2006)

Primer name	*Sequence*
P*ydaM*-*Eco*RI	5′-CG***GAATTC***AGAATTATCTGATCATATGACGTGG-3′
P*ydaM*-*Hind*III	5′-GGCC***AAGCTT***TTATGCCGCCAGCACGCGGTTGC-3′

Inside mutagenesis primers

Primer name	*Sequence*
P*ydaM*-EE/AA-f	5′-CGTTGGGGAGGCG**C**AG**C**GTTTGTCTTATTGC-3′
P*ydaM*-EE/AA-r	5′-TAAGACAAAC**G**CT**G**CGCCTCCCCAACG-3′

Tab. 3.6 (continued)

XI. Primers for mutagenizing and cloning the mutated *mlrA* and *yciR* promoters into pJL28		
Outside/cloning primers (constructed by Athanasios Typas)		
Primer name		*Sequence*
pJL-upstream		5′-ACCAGCGTTTCTGGGTGAGC-3′
lacZ_u_110		5′-CGCCAGCTGGCGAAAGGG-3′
Inside mutagenesis primers		
Mutation	*Primer name*	*Sequence*
mlrA-T-6C	P*mlrA*-T6C-for P*mlrA*-T6C-rev	5′-CTGCGTCTAAAGT**C**AAACCGGGACCTC-3′ 5′-GAGGTCCCGGTTT**G**ACTTTAGACGCAG-3′
mlrA-T-7A	P*mlrA*-T7A-for P*mlrA*-T7A-rev	5′-CTGCGTCTAAAG**A**TAAACCGGGACCTC-3′ 5′-GAGGTCCCGGTTTA**T**CTTTAGACGCAG-3′
mlrA-T-12A	P*mlrA*-T12A-for P*mlrA*-T12A-rev	5′-CTGCGTC**A**AAAGTTAAACCGGGAC-3′ 5′-GTCCCGGTTTAACTTT**T**GACGCAG-3′
mlrA-C-13G	P*mlrA*-C-13G-for P*mlrA*-C-13G-rev	5′-GCAAAACTGCGT**G**TAAAGTTAAACCGGG ACC-3′ 5′-GGTCCCGGTTTAACTTTA**C**ACGCAGTTTT GC-3′
mlrA-TC-14/-13GG	P*mlrA*-TC1314GG-for P*mlrA*-TC1314GG-rev	5′-GCAAAACTGCG**GG**TAAAGTTAAACCGGGA CC-3′ 5′-GGTCCCGGTTTAACTTTA**CC**CGCAGTTTT GC-3′
mlrA-AA-22/-21TT	P*mlrA*-AA22/2TT-for P*mlrA*-AA22/2TT-rev	5′-CCTGGTTCGCA**TT**ACTGCGTC-3′ 5′-GACGCAGT**AA**TGCGAACCAGG-3′
mlrA-C-24T	P*mlrA*-C24T-for P*mlrA*-C24T-rev	5′-CCTGGTTCG**T**AAAACTGCGTC-3′ 5′-GACGCAGTTTT**A**CGAACCAGG-3′
yciR-C-23T	PyciR-CT-for PyciR-CT-rev	5′-CTGGCGTTTTTC**T**AAAACTGGATTAC-3′ 5′-GTAATCCAGTTTT**A**GAAAAACGCCAG-3′

XII. Primers for generating DNA-fragments used for electrophoretic mobility shift assays		
EMSA-fragment	*Primer name*	*Sequence*
chaB	P*chaB*-for P*chaB*-rev	5′-CAGAAAGTGTCTGGATATCG-3′ 5′-CGGTAGAACGTGCTTTACGC-3′
csgD	FMO49 FMO50, constructed by Franziska Mika)	5′-CACCGAAATATTTTTTATATGC-3′ 5′-CAATCTAGCCATTACAAATCTTA-3′
flgA	*flgA*1 (*Bam*HI) *flgA*2 (*Hind*III) (Barembruch and Hengge 2007)	5′-CTGGGATCCGCTTAAATGCCTTTAC-3′ 5′-GCCAAGCTTCGTTTTATTATCAGC-3′
flgM	*flgM*3 (*Bam*HI) *flgM*4 (*Hind*III) (Barembruch and Hengge 2007)	5′-CATGGATCCGGGACAGGTAGTCA GCG-3′ 5′-GAACGAAGCTTACAGGCTTCAGA GG-3′
flhDC-long	P*flhD*-GS-for P*flhD*-GS-rev	5′-GACTGAGTCAGCCGAGAAG-3′ 5′-GCTGCAATAAGCAGAACCACC-3′
flhDC-short	P*flhD*-GS-for Pf*lhD*-GS-no_pu_pro-rev	5′-GACTGAGTCAGCCGAGAAG-3′ 5′-CCTAAATCGACGCAACTGTAC-3′
gadB	P*gadB*-GS-for P*gadB*-GS-rev	5′-TATTCGCGTAATATCTCACG-3′ 3′-GTGGAAATAGACTTCGCACC-3′
gadE	P*gadE*-for P*gadE*-rev	5′-CAAGCTGATAACAACCAGG-3′ 5′-CTTTCAACTGCCAAAAGCCCTG-3′
gatY	P*gatY*-for P*gatY*-rev	5′-CACGCGCACTTTGCTACGGC-3′ 5′-ATATTGAATGCCGGAACCGC-3′

Tab. 3.6 (continued)

EMSA-fragment	*Primer name*	*Sequence*
hdeA	P*hdeA*-for P*hdeA*-rev	5′-CGCGTCTAAGAATGCAGTCG-3′ 5′-GCATTGCTCACAACTGGCAG-3′
malE	P*malE*-for P*malE*-rev	5′-GGAATTTCGTGATGTTGCTTGC-3′ 5′-GGCGGAAAACATCATCGTCG-3′
malK	P*malK*-for P*malK*-rev	5′-GCGCACATAAAATCGCCACG-3′ 5′-ATACCACGACCTCGCCCCAG-3′
mlrA-wt & mutated promoters	*mlrA*-up-125 FMO15, constructed by Franziska Mika)	5′-CGATCACTCAAAATCGCCTGG-3 5′-TAACGCCTCTGCCACGCGCGTAACG-3′
mlrA-TR	GS-*mlrA*-TR-for GS-*mlrA*-TR-rev	5′-ATGGCGCTTTACACAATTGGTG-3′ 5′-CAGGCTATGTAGATTGCCGCTTTGC-3′
nsrR	P*nsrR*-gelshift-up P*nsrR*-gelshift-down	5′-GGATCGTACTGAAACCATGATTC-3′ 5′-ACCAGCCACAAGCTGTGTCG-3′
rpoS	FMO9 FMO10 (both primers constructed by Franziska Mika)	5′-ACGTTGGTCAGACCTTGCAGGT-3′ 5′-TACTGGTTGATGTACTGCTGA-3′
xerD	P*xerD*-for P*xerD*-rev	5′- TGTAACAGGTGCTGGAACCG-3′ 5′- CACTCCACCATCATTGACAG-3′
yciR-wt & mutated promoters	P*yciR*-fp-new-for PyciR-fp-new-rev	5′-GCGCGCCCGGTCGCGTAATCTCCTT TCACG-3′ 5′-GACTTACATGAAATTAACGGCGGC TAA ACGC-3′
yjcC	P*yjcC-EcoR*I P*yjcC-Hind*III (Sommerfeldt et al. 2009)	5′-CGGAATTCCACAATTGATTGTTTGTTA GCC-3′ 5′-CCCAAGCTTCCGGCAACGCCAGTAAT TGG-3′
ylaB	P*ylaB-EcoR*I P*ylaB-Hind*III (Sommerfeldt et al. 2009)	5′-CGGAATTCCTGCCGCGCTGGTTGAA GC-3′ 5′-CCCAAGCTTGGCCGACCAGATGTCG TG-3′
yjbJ	P*yjbJ*-for P*yjbJ*-rev	5′-GGTTTGCCGCAACGTGACGG-3′ 3′-CGTCATATCATCATCGGTC-3′
ynhG	P*ynhG*-for P*ynhG*-rev	5′-GCCGCTGCACTTAGCTAAAC-3′ 5′-GCGGATAATCAACCGCCCAG-3′

XIII. Primers for generating DNA-fragments for DNaseI footprint assays with FliZ

Promoter	*Primer name*	*Sequence*
flhDC	P*flhD*-fp-for P*flhD*-fp-rev-dig	5′-TTGTGTGATCTGCATCACGC-3′ 5′-DIG-AGTTGCGATAAGCTGCAATAAGC-3′
gadE	P*gadE*-fp-for-dig P*gadE*-rev	5′-DIG-GTTCACGAAGGGTAAAGTTC-3′ see above
hdeA	P*hdeA*-fp-for-dig P*hdeA*-rev	5′-DIG-GATGCATCTGTAACTCATTG-3′ see above
mlrA	P*mlrA*-up-dig FMO15, constructed by Franziska Mika)	5′-DIG-CGATCACTCAAAATCGCCTGG-3′ 5′-TAACGCCTCTGCCACGCGCGTAACG-3′
yciR	P*yciR*-fp-new-for P*yciR*-fp-new-rev-dig	5′-GCGCGCCCGGTCGCGTAATCTCCTTTC ACG-3′ 5′-DIG-GACTTACATGAAATTAACGGCGGC TAAACGC-3′

Tab. 3.6 (continued)

XIV. Primers for generating DNA-fragments for DNaseI footprint assays with MlrA

DNA fragment	*Primer name*	*Sequence*
region upstream of *csgD* promoter (including sequence resembling a σ^S-dependent promoter)	P*csgD*-prom-fp-for	5′-CCAAATGTACAACTTTTCTATCATTTC-3′
	P*csgD*-prom-fp-rev-Dig	5′-Dig-ATGTTGCACTGCTGTGTGTAG-3′
csgD promoter	P*csgD*-crypt.prom-fp-for	5′-CGTTTTACATGACGAAAGGAC-3′
	P*csgD*-crypt.prom-fp-rev-Dig	5′-Dig-AATCAAGTGTTAAACATGTAACTAAATG-3′

XV. Primers for determining the 5′-end of *yciR* mRNA by primer extension:

Labelled primer	
Primer name	*Sequence*
P*yciR*-*Hind*III (generated in Diploma thesis, 2005)	5′- CCCAAGCTTGCCAGTATGGATTGTGCGATCCGAG-3′
Forward primer for generating template for sequencing reaction	
Primer name	*Sequence*
P*yciR*-fp-up	5′-CCATTCCTCATGGATGGGCCG-3′

XVI. Primers for generating probes for Northern blot analysis:

Gene	*Primer name*	Sequence
fliZ	P*fliZ*-northern-for	5′-CACAGCCAGACCCATTGCGC-3′
	P*fliZ*-northern-rev	5′-TCACTCTGCGTTTTGCAATG-3′
fliY	P*fliY*-northern-for	5′-GGCGCTGGTTGCGGGCATGAGC-3′
	P*fliY*-northern-rev	5′-GTGACGCCTCAACGCCAAGATG-3′
ymgB	*ymgB*-for-sonde	5′-ATGCTTGAAGATACTACAATTC-3′
	ymgB-rev-sonde (both primers constructed by Natalia Tschowri)	5′-TTACATATCATCAGCTGTGTATC-3′

4. Results

4.1 Inverse coordination of motility and curli fimbriae-mediated adhesion in *E. coli*

E. coli cells grown in nutrient-rich medium undergo a lifestyle transition from the planktonic, single-cell lifestyle of highly motile cells during post-exponential phase to the sedentary, adhesive lifestyle of stationary phase cells, which have lost motility and express adhesive structures, such as curli fimbriae (Adler and Templeton 1967; Amsler et al. 1993; Arnqvist et al. 1994). This growth phase-dependent succession of motility and curli fimbriae-mediated adhesion was a first indication that the systems controlling motility and curli fimbriae expression do not function independently of each other, but may be connected through regulatory links that establish mutual exclusion and inverse coordination of these two lifestyle features. The analyses that will be presented in the first part of the results chapter were aimed at the identification of such regulatory links between the two cascades driving flagellar motility and curli fimbriae expression, with a focus on the influence that flagellar gene expression exerts on curli fimbriae expression, i.e. on regulatory links that originate from the motility system and are directed at the curli control system.

4.1.1 Permanent induction of the flagellar gene regulon inhibits curli fimbriae expression

As a first test, whether the expression of flagella affects curli fimbriae expression in the *E. coli* strain MC4100, the motility defect of this non-motile strain was complemented by expression of the flagellar master regulator operon *flhDC* from a low copy plasmid (pCAB19). Upon induction with IPTG, p_{tac} promoter-driven expression of *flhDC* from this plasmid establishes similar levels of flagellar gene products as observed in a motile MC4100 derivative carrying an intact *flhDC* sequence (obtained by P1 transduction), and restores motility (Barembruch 2007; Barembruch and Hengge 2007). Since curli fimbriae expression is temperature-regulated and in most strains only induced at temperatures below 30°C (Olsen et al. 1989; Arnqvist et al. 1992), all experiments were conducted at 28°C. Interestingly, ectopic *flhDC* expression completely abolished curli fimbriae expression as observer using a

MC4100 derivative carrying a single copy chromosomal *lacZ* fusion to the *csgB* gene, which is the first gene in the operon encoding the curli structural components (Fig. 4.1A).

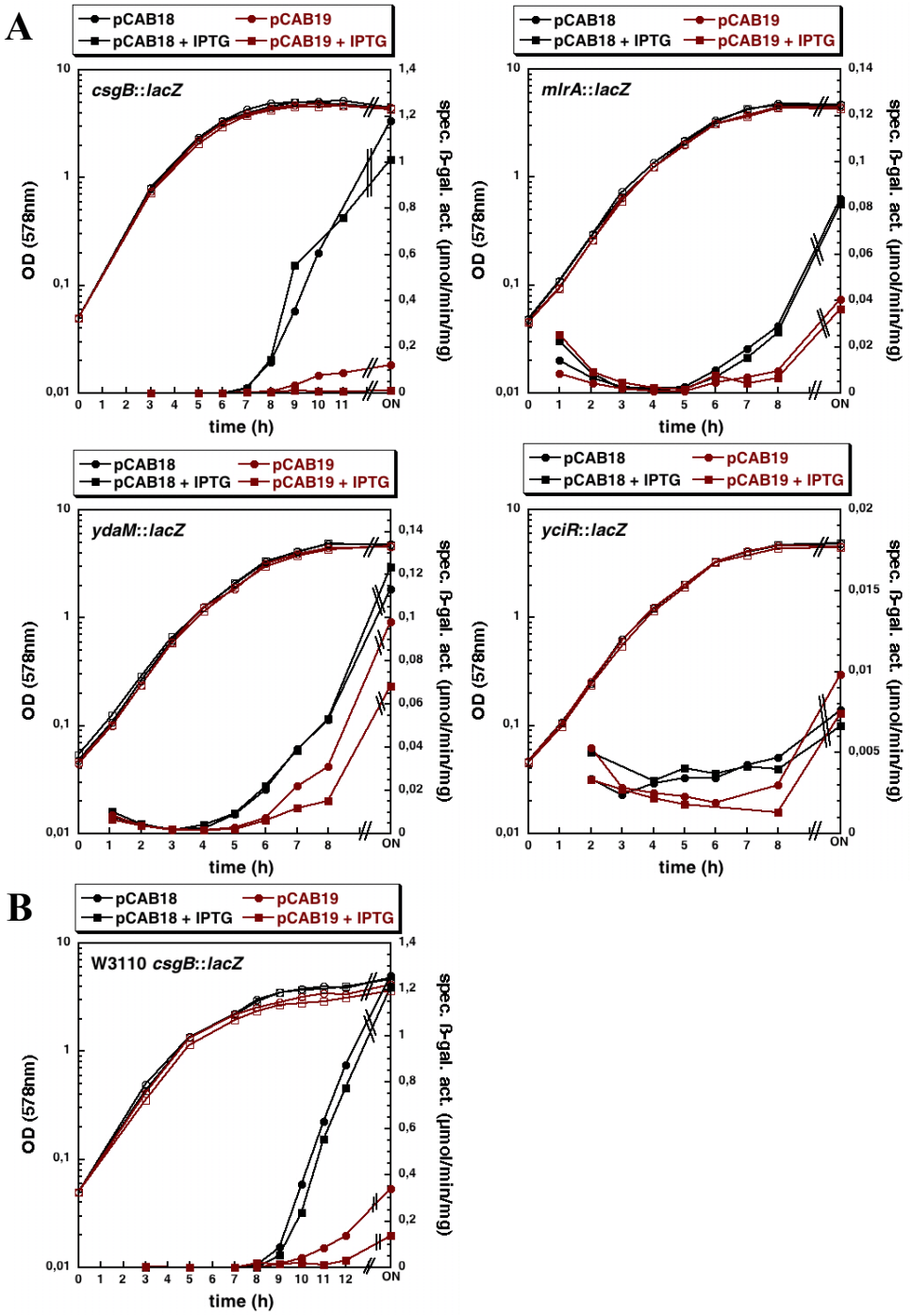

Fig. 4.1: Ectopic expression of the flagellar gene regulon inhibits curli fimbriae expression. Expression of single copy chromosomal *lacZ* fusions to the indicated curli structural and regulatory genes in (A) MC4100 and (B) W3110 derivatives carrying either the empty low copy plasmid pCAB18 or its derivative pCAB19, expressing the *flhDC* operon under p_{tac} promoter control. Cells were grown at 28°C in LB medium supplemented with ampicillin in the presence or absence of 10 µM IPTG to induce *flhDC* expression as indicated. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in the overnight cultures (ON).

Moreover, expression of several genes coding for regulators of curli fimbriae expression, i.e. the MerR-like transcriptional regulator MlrA, the diguanylate cyclase YdaM, which is essential for curli fimbriae expression, and its antagonist, the phosphodiesterase YciR, was also reduced in strains expressing *flhDC* from the low copy plasmid (Fig. 4.1A), thus indicating a comprehensive inhibitory effect of flagellar gene expression on the curli control cascade that results in shut-down of curli fimbriae expression.

In contrast to strain MC4100, the *E. coli* K12 strain W3110, another commonly used strain, carries an intact *flhDC* allele and shows the above mentioned growth phase-dependent succession of motility and adhesion. Experiments performed in the Hengge group had demonstrated that W3110 shows normal motility and chemotactic behaviour when inoculated into soft agar plates and that stationary-phase induced expression of curli fimbriae is under the control of the same regulators (σ^S, MlrA, CsgD, YdaM and YciR) as in MC4100 (Gisela Becker in (Pesavento et al. 2008)). Thus, in contrast to repression of curli fimbriae expression in the presence of ectopically expressed *flhDC* in MC4100, endogenous expression of chromosomally encoded *flhDC* does not seem to interfere with curli expression in this strain. However, expression of *flhDC* from pCAB19 also eliminated curli fimbriae expression in this strain (Fig. 4.1B), suggesting a regulatory difference between strains expressing the flagellar master regulator from a single chromosomal copy of the operon under the control of the natural promoter and strains expressing it from the pCAB19 plasmid. This difference most likely lies in the continuous induction of *flhDC* expression from the plasmid, which is in contrast to the decline in endogenous flagellar gene expression observed in strains entering stationary phase ((Adler and Templeton 1967; Amsler et al. 1993) and see Fig. 4.6)). Thus, the shut-down of flagellar gene expression seems to be a prerequisite for successful induction of curli fimbriae expression upon entry into stationary phase. Together, these initial experiments indicated that motility gene expression indeed interferes with curli fimbriae expression and that the flagellar regulon includes one or several inhibitor(s) of curli fimbriae expression.

4.1.2 The flagellar protein FliZ is the key regulator responsible for repression of curli fimbriae expression

4.1.2.1 Identification of FliZ

The flagellar regulon in *E. coli* comprises more than 60 genes. In order to identify the regulator(s) responsible for inhibition of curli fimbriae expression upon induction of flagellar

gene expression, a screen based on the strong repression of *csgB::lacZ* expression in the presence of *flhDC* expression from plasmid pCAB19 was used. First, the effect of a mutation in *fliA*, encoding the flagellar sigma factor σ^{28} (FliA), on expression of *csgB::lacZ* in cells expressing *flhDC* from pCAB19 was tested. A *fliA::cat* mutation completely abolished pCAB19-mediated repression of *csgB::lacZ* expression (Fig. 4.2A).

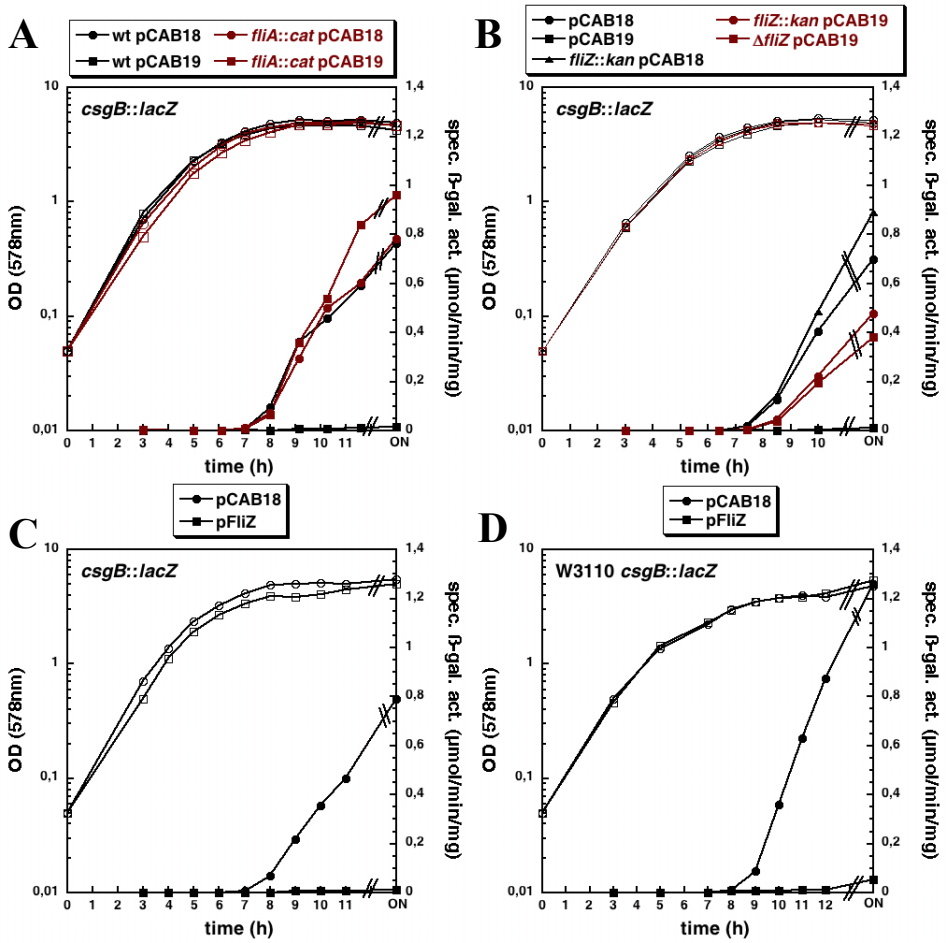

Fig. 4.2: Identification of FliZ as the flagellar protein mainly responsible for inhibition of curli fimbriae expression. Expression of a single copy chromosomal *csgB*::*lacZ* fusions in **(A & B)** MC4100 wild-type (wt) and flagellar gene mutant derivatives that carry the empty low copy plasmid pCAB18 or its derivative pCAB19, expressing the *flhDC* operon under p_{tac} promoter control, and in **(C)** MC4100 and **(D)** W3110 derivatives that carry pFliZ, expressing the single flagellar *fliZ* gene under p_{tac} promoter control. Cells were grown at 28°C in LB medium supplemented with ampicillin in the presence (A & B) or absence (C & D) of 10 µM IPTG. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in the overnight cultures (ON).

Based on this observation, three possible scenarios could be envisaged: Firstly, a σ^{28}-dependent flagellar class III gene product may constitute the sought-after inhibitor of curli fimbriae expression. Alternatively, σ^{28} itself may be the inhibitor, as artificially prolonged expression of σ^{28} in stationary phase might interfere with σ^{S} activity and therefore curli fimbriae synthesis due to competition for free RNA polymerase core enzyme. Finally, the

fliA::cat mutation might exert a polar effect on the two downstream genes in the *fliAZY* operon, suggesting FliZ and FliY, two proteins with largely uncharacterized functions, as possible regulators mediating curli repression. To test the last hypothesis, *csgB::lacZ* expression in the presence of ectopic *flhDC* expression was analyzed in polar (*fliZ::kan*) and non-polar (Δ*fliZ*) *fliZ* mutants (Fig. 4.2B). Both *fliZ* mutations partially suppressed inhibition of curli fimbriae expression. Since both *fliZ* mutants still express the flagellar sigma factor σ^{28}, this indicated that neither σ^{28} nor any flagellar class III gene product encoded outside of the *fliAZY* operon was solely responsible for the strong repression of *csgB::lacZ* expression upon *flhDC*-induction. Moreover, both the polar *fliZ::kan* mutant, which does not express the downstream gene *fliY*, and the non-polar *fliZ* mutant, in which *fliY* is expressed, restored curli fimbriae expression to similar extents. Thus, participation of FliY in curli fimbriae repression seemed unlikely. Together these results strongly suggested that FliZ represents the flagellar inhibitor of curli fimbriae expression.

To further corroborate this hypothesis, *fliZ* alone was expressed from the same low copy vector used for induction of *flhDC* expression. MC4100 cells expressing *fliZ* from this plasmid (pFliZ) generate no other flagellar gene products besides plasmid-encoded FliZ, due to the frameshift mutation in *flhD*. pFliZ completely eliminated curli fimbriae expression in both MC4100 (Fig. 4.2C) and W3110 (Fig. 4.2D), even in the absence of the inducer IPTG, thus identifying FliZ as a potent inhibitor of curli fimbriae expression.

In order to analyse on which level FliZ interferes with curli fimbriae control, expression of several genes coding for regulators of the curli control cascade was monitored in the presence of pFliZ. Analogous to the situation in the strain expressing *flhDC* form pCAB19 (Fig. 4.1 A), the presence of pFliZ strongly repressed expression of MlrA, YdaM and YciR (Fig. 4.3A-C). This inhibitory effect of pFliZ on expression of curli control genes could also be observed at 37°C (Fig. 4.3D), indicating that FliZ is not involved in temperature control of curli fimbriae expression. MlrA, YdaM and YciR specifically control the transcription of the essential curli regulator CsgD (Weber et al. 2006) and in accordance with down-regulation of this control module, CsgD levels were strongly reduced in cells ectopically expressing *flhDC* and almost eliminated in strains carrying pFliZ (Fig. 4.3E). In contrast to CsgD, σ^S levels were not altered in the presence of ectopic *flhDC* and *fliZ* expression (Fig. 4.3F), indicating that FliZ interferes with curli fimbriae expression on a level downstream of σ^S expression.

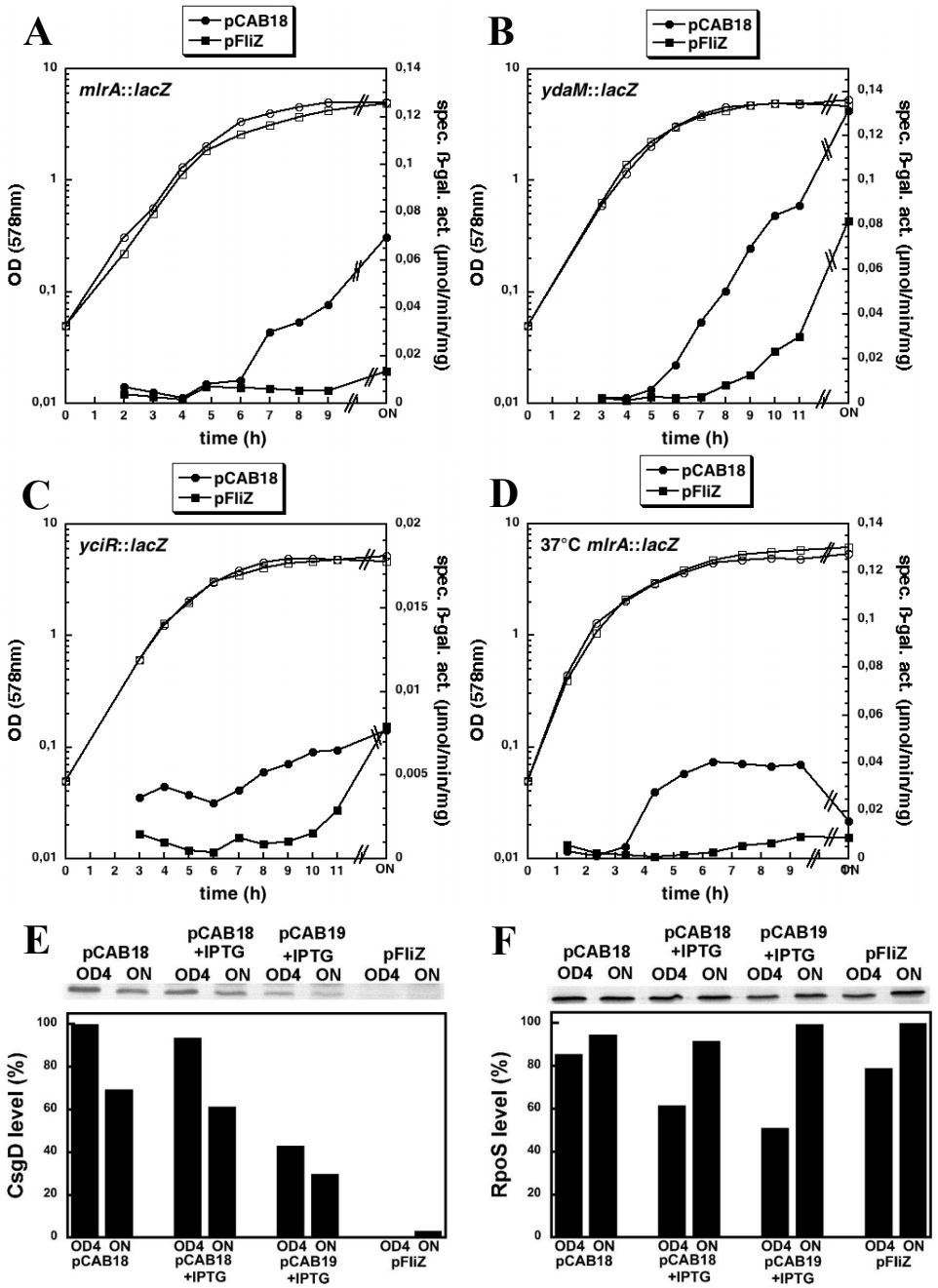

Fig. 4.3: FliZ is a potent inhibitor of curli control genes, but does not interfere with σ^S levels. (A-D) MC4100 derivatives carrying single copy chromosomal *lacZ* fusions to the indicated curli control genes and either the empty low copy plasmid pCAB18 or its derivative pFliZ, expressing *fliZ* under p_{tac} promoter control in the absence of inducer. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in the overnight cultures (ON) with cells grown at 28°C (A-C) or 37°C (D) in LB medium supplemented with ampicillin; **(E & F)** Immunoblot analysis of CsgD (E) and σ^S (RpoS) (F) levels in MC4100 derivatives carrying either the empty low copy plasmid pCAB18 or its derivatives pCAB19 or pFliZ expressing *flhDC* or *fliZ*, respectively, under p_{tac} promoter control in the presence and absence of IPTG as indicated. Cells were grown at 28°C in LB medium supplemented with ampicillin. Samples were taken at an OD_{578nm} of 4 and in the overnight cultures (ON). Densitometric quantification is shown below the blots.

4.1.2.2 FliZ interferes with σ^S-dependent gene expression

Since all FliZ-repressed genes in the curli regulatory cascade are controlled by σ^S, repression of these genes in the presence of unchanged σ^S levels suggested that FliZ might interfere with σ^S activity, thereby exerting a strong influence on curli fimbriae expression, which is under multiple feedforward control of σ^S. Potential interference with σ^S activity raised the possibility that FliZ does not only interfere with expression of the σ^S-dependent curli control genes, but has a more general effect on the σ^S regulon. In accordance with this hypothesis, expression of two σ^S-dependent genes not associated with curli fimbriae formation, i.e. *osmY* and *gadB*, was also repressed in strains carrying pFliZ (Fig. 4.4). Repression of the *gadB* gene was particularly strong. Like *csgB*, *gadB* expression is controlled by a cascade under multiple feedforward control of σ^S.

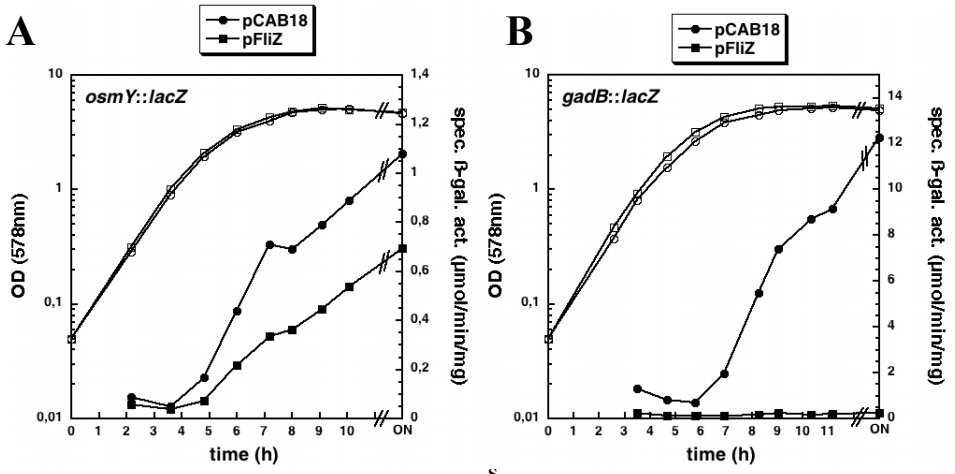

Fig. 4.4: FliZ interferes with the expression of σ^S-dependent genes not associated with curli fimbriae formation. Expression of single copy chromosomal *lacZ* fusions to the σ^S-dependent genes *osmY* **(A)** that encodes a protein involved in the hyperosmotic stress response and *gadB* **(B)** that codes for a protein involved in the acid stress response in MC4100 derivatives carrying either the empty low copy plasmid pCAB18 or its derivative pFliZ, expressing the *fliZ* gene under p_{tac} promoter control. Cells were grown at 28°C in LB medium supplemented with ampicillin. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in the overnight cultures (ON).

To confirm this general effect of FliZ on σ^S-dependent gene expression, microarray experiments were performed to compare the genome wide transcription profile of stationary phase cells expressing *fliZ* from the low copy plasmid with that of cells carrying the empty vector. The identified genes that were differentially regulated in the presence of pFliZ are listed in table 4.1, which shows that both positively and negatively FliZ-regulated genes were found. Strikingly, the majority of genes repressed by FliZ had previously been shown to belong to the σ^S regulon ((Weber et al. 2005) and unpublished results by Harald Weber and Regine Hengge for cells grown at 28°C) (Tab. 4.1). In addition, there was a strong overlap

between genes repressed by FliZ and genes positively regulated by Crl (Typas et al. 2007a) (Tab. 4.1). Crl is a regulator that specifically helps σ^S in its competition for core enzyme, thereby positively influencing σ^S activity (Typas et al. 2007a). This suggested that FliZ assumes a functionally antagonistic role to Crl in regulation of σ^S.

These microarray studies strongly support that FliZ exerts a general effect on σ^S-dependent gene expression by interfering with σ^S activity. A detailed analysis of the mechanism employed by FliZ to mediate this comprehensive effect on σ^S-dependent gene expression will be given in section 4.2 of this work. The following parts will focus on a more detailed characterization of the physiological role of FliZ, on the regulation of FliZ itself and on other mechanisms that are integrated with FliZ-mediated regulation to inversely regulate motility and curli fimbriae expression.

Tab. 4.1: FliZ-controlled genes. Genes identified by comparing whole-genome transcription profiles of MC4100 derivatives carrying either pFliZ or the empty vector pCAB18. Cells were grown in LB medium at 28°C without inducer and harvested at an OD_{578nm} of 4. FliZ-regulated genes are listed in alphabetical order with their b-numbers and a short description of molecular or physiological functions. **(A)** Expression ratios (pFliZ-carrying strain/pCAB18-carrying strain) of positively FliZ-dependent genes **(B)** expression ratios (pFliZ-carrying strain/pCAB18-carrying strain) of negatively FliZ-dependent genes **(C)** genes that have previously been shown to be σ^S-dependent are indicated by "x", and "28" indicates genes that show σ^S dependence only at 28°C, **(D)** genes that have previously been shown to be Crl-dependent are indicated by "x". The table only includes genes with ratios > 3 for positively FliZ-dependent genes and ratios < 0.33 for negatively FliZ-dependent genes.

Name	ID	Description	A	B	C	D
acrB	b0462	acridine efflux pump	6,132			
allA	b0505	ureidoglycolate amidohydrolase; allantoin assimilation	5,069			
allB	b0512	allantoinase; allantooin assimilation	10,663			
arpB2	b1721	orf, hypothetical protein	13,419			
chaB	b1217	cation transport regulator	10,266		x	
csgA	b1042	curlin major subunit		0,052	x	x
csgB	b1041	curli nucleator		0,029	x	x
csgD	b1040	curli transcriptional activator		0,111	x	
csgE	b1039	curli production assembly/transport component		0,146	x	
csgF	b1038	curli production assembly/transport component		0,166	x	
csgG	b1037	curli production assembly/transport component		0,116	x	
dsbC	b2893	protein disulfide isomerase II		0,091		
fliZ	b1921	FliZ	46,552			
gadB	b1493	glutamate decarboxylase isozyme		0,103	x	x
gadC	b1492	acid sensitivity protein, putative transporter		0,243	x	x
gadE	b3512	transcriptional activator		0,091	x	x
gatA	b2094	galactitol-specific enzyme IIA of phosphotransferase		0,122		
gatB	b2093	galactitol-specific enzyme IIB of phosphotransferase		0,161		
gatZ	b2095	subunit of tagatose-1,6-bisphosphate aldolase 2		0,196		
gcl	b0507	glyoxylate carboligase	12,870			
glpF	b3927	glycerol MIP channel, facilitated diffusion of glycerol	3,675			
glxR	b0509	putative oxidoreductase	11,008			
glyS	b3559	glycine tRNA synthetase, beta subunit		0,278		
gyrA	b2231	DNA gyrase, subunit A, type II topoisomerase		0,239		
hdeA	b3510	acid resistance protein, chaperone		0,014	x	x
hdeB	b3509	acid stress chaperone		0,040	x	x
hsdM	b4349	DNA methylase M; host modification	4,143			
hyi	b0508	hydroxypyruvate isomerise, glyoxylate-induced protein	11,130			

Tab. 4.1 (continued)

Name	ID	Description	A	B	C	D
lamB	b4036	phage lambda receptor pr.; maltose high-affinity receptor	6,115			
lrp	b0889	transcriptional dual regulator		0,162		
malE	b4034	periplasmic maltose-binding protein	4,171			
malK	b4035	ATP-binding component of transport system for maltose	8,250			
malM	b4037	periplasmic protein of *mal* regulon	4,314			
modE	b0761	molybdate uptake regulatory protein	3,732			
mokB	b1420	regulatory peptide, translation enables *hokB* expression	5,239			
narG	b1224	nitrate reductase 1, alpha subunit	7,493			
ompC	b2215	outer membrane porin C		0,111	28	
oppA	b1243	oligopeptide transport; periplasmic binding protein		0,180		
priC	b0467	primosomal replication protein N"	3,934			
putA	b1014	proline dehydrogenase, P5C dehydrogenase	6,551			
puuA	b1297	γ-glutamylputrescine synthetase		0,222		
rzpQ	b1573	Qin prophage; predicted protein	3,789			
tdcC	b3116	TdcC threonine STP transporter		0,041	28	x
treB	b4240	PTS system enzyme II, trehalose specific	10,608			
ybbW	b0511	uncharacterized member of NCS1 transporter family	19,557			
ycfZ	b1121	homolog of virulence factor	14,587			
ydfA	b1571	Qin prophage; predicted protein	27,483			
ydfB	b1572	Qin prophage; predicted protein	14,183			
ydiT	b1700	putative ferredoxin		0,219		
yhjR	b3535	conserved protein		0,269	28	x
yiaM	b3577	predicted transporter		0,168		
yjbJ	b4045	predicted stress response protein		0,103	x	x
ymfE	b1138	orf, hypothetical protein		0,116		x
ymgG	b1172	predicted protein	4,675			
ynhG	b1678	conserved protein	3,626		x	
yoaI	b1788	predicted protein	9,572			

4.1.2.3 The role of FliZ in the inverse coordination of motility and curli fimbriae expression is to act as a timing device in curli fimbriae expression

For a more detailed analysis of the physiological function of FliZ, the *E. coli* strain W3110 was used because, as stated above, this motile strain expresses the flagellar gene regulon, thus allowing for the analysis of *fliZ* mutations in a physiological context.

As shown above, FliZ interferes with expression of the curli control module consisting of the regulators MlrA, YdaM and YciR. The diguanylate cyclase YdaM and the transcriptional activator MlrA are essential factors for activation of curli fimbriae expression (Weber et al. 2006). While expression of *ydaM* is induced at an OD_{578nm} of approximately 2, *mlrA* expression starts rather late, around an OD_{578nm} of approximately 3 (compare Fig. 4.3A and B). Expression of both genes starts later than expression of some σ^S-dependent genes with high affinity promoters such as e.g. *osmY* (Fig. 4.4A). The induction of the latter seems to directly follow accumulation of σ^S which starts around an OD_{578nm} of approximately 1 in rich medium (Lange and Hengge-Aronis 1994), while expression of *ydaM* and *mlrA* seems to be delayed with respect to accumulation of σ^S. Interestingly, this “delay” in *mlrA* expression was

abolished in a W3110 *fliZ* mutant that induced *mlrA* expression already in post-exponential phase, almost two hours earlier than the wild-type, i.e. around an OD_{578nm} of 1 (Fig. 4.5A).

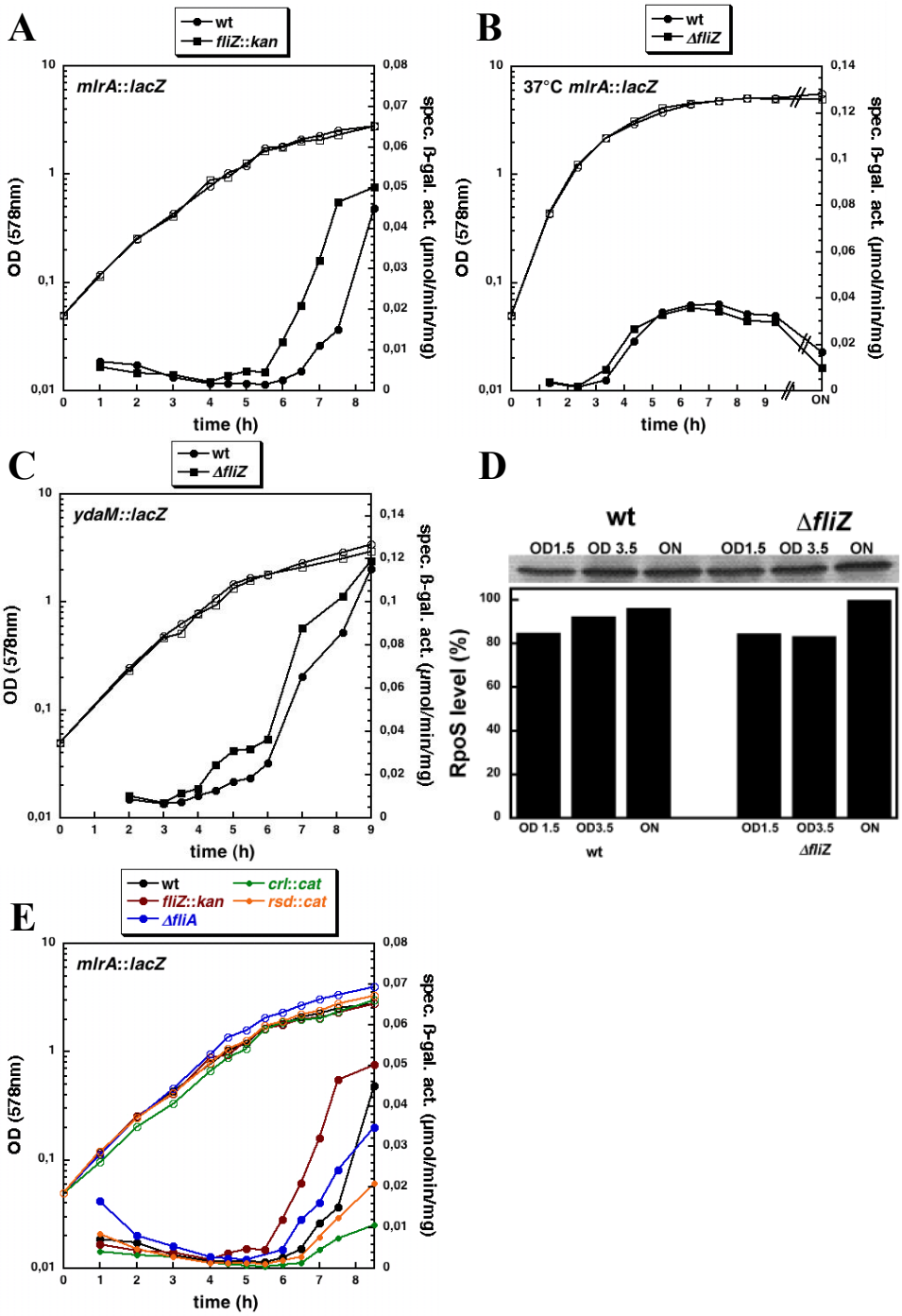

Fig. 4.5: FliZ acts as a timing device for curli fimbriae expression by transiently repressing curli control genes during post-exponential phase. (A-C, E) Expression of single copy chromosomal *lacZ* fusions to the indicated curli control genes in W3110 wild-type (wt) and mutant derivatives carrying mutations in (A-E) *fliZ* or (E) genes that are known to affect the sigma factor competition balance and $E\sigma^S$ levels during entry into stationary phase. Cells were grown at 28°C (A, C, D) or 37°C (B) in LB medium as indicated. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in the overnight cultures (ON). The experiment shown in figure E reproduced results shown by Aylin Kademci in her diploma thesis, 2007. **(D)** Immunoblot analysis of σ^S (RpoS) levels in W3110 wild-type (wt) and Δ*fliZ* mutant derivatives. Cells were grown at 28°C in LB medium and samples were taken at an OD_{578nm} of 1.5, 3.5 and in the overnight culture (ON). Densitometric quantification is shown below the blots.

Slightly but reproducibly earlier induction of *mlrA* expression could also be observed at 37°C, again indicating that FliZ-mediated regulation is independent of temperature (Fig. 4.5B). Moreover, expression of *ydaM* also started slightly earlier in the W3110 Δ*fliZ* mutant (Fig. 4.5C). Thus, FliZ seems to transiently repress the expression of these σ^S-dependent genes during post-exponential phase, i.e. when flagellar gene expression peaks. Since σ^S levels were unaltered in the Δ*fliZ* mutant as compared to the wild-type (Fig. 4.5D), this confirmed that FliZ affects the activity of σ^S rather than its expression or stability.

With MlrA being the latest one of the essential curli regulators to be induced, its expression determines the timing of curli fimbriae expression. Comparison of *mlrA* expression in strains carrying different mutations that alter levels of σ^S-containing RNAP holoenzyme and therefore σ^S activity showed that timing of *mlrA* expression seems to be very sensitive to these alterations (Fig. 4.5E). In post-exponential phase the flagellar sigma factor σ^{28} (FliA) competes with σ^S for RNAP core enzyme. Consistently, a mutation in *fliA* resulted in earlier induction of σ^S-dependent *mlrA* expression. Conversely, mutations in *rsd*, which encodes an anti-σ^{70} factor and *crl*, which codes for a stimulator of $E\sigma^S$ formation, result in negative effects on the ability of σ^S to compete for RNAP core enzyme. These mutations caused a delay in *mlrA* expression. Together these observations strongly indicate that FliZ acts as a timing device that transiently inhibits expression of genes belonging to the σ^S regulon during post-exponential phase, most likely by interfering with σ^S activity. Genes like *mlrA*, which are highly sensitive to alterations in $E\sigma^S$ levels and activity in their timing of induction, seem to be most strongly affected by FliZ.

4.1.3 The balance between sigma factors involved in regulation of flagella and curli fimbriae expression contributes to inverse regulation of motility and adhesion

As described above, introduction of the *fliA::cat* mutation into the strain ectopically expressing *flhDC* from the low copy plasmid completely suppressed inhibition of *csgB::lacZ* expression (Fig. 4.2A). The strong inhibitory effect was essentially attributed to FliZ-mediated inhibition of σ^S activity, however, in contrast to complete suppression in the *fliA::cat* mutant, curli repression was only partially suppressed in the *fliZ* mutant (compare Fig. 4.2 A and B). This indicated that in addition to FliZ, the flagellar sigma factor σ^{28} (FliA) might also contribute to the inhibition of curli fimbriae expression by the flagellar system.

Consistent with this hypothesis, introduction of a non-polar *fliA* mutation (Δ*fliA*), which still allows for expression of *fliZ*, also lead to partial suppression of *csgB::lacZ* expression in the strain expressing *flhDC* from pCAB19 (Fig. 4.6A). This observation suggested that artificially prolonged expression of σ^{28} in stationary phase (caused by p_{tac}-driven *flhDC* expression) interferes with σ^S-controlled curli fimbriae expression, possibly due to increased competition for limiting amounts of RNAP core enzyme. A role for sigma factor competition between σ^{28} and σ^S in the coordination of motility and curli gene expression is also supported by the above mentioned earlier induction of *mlrA* expression in *fliA* mutants (see Fig. 4.5E). Alternatively, prolonged expression of a flagellar class III gene product that exerts an inhibitory effect on curli fimbriae expression (e.g. YhjH, see 4.1.5) might mediate this σ^{28}-dependent repression.

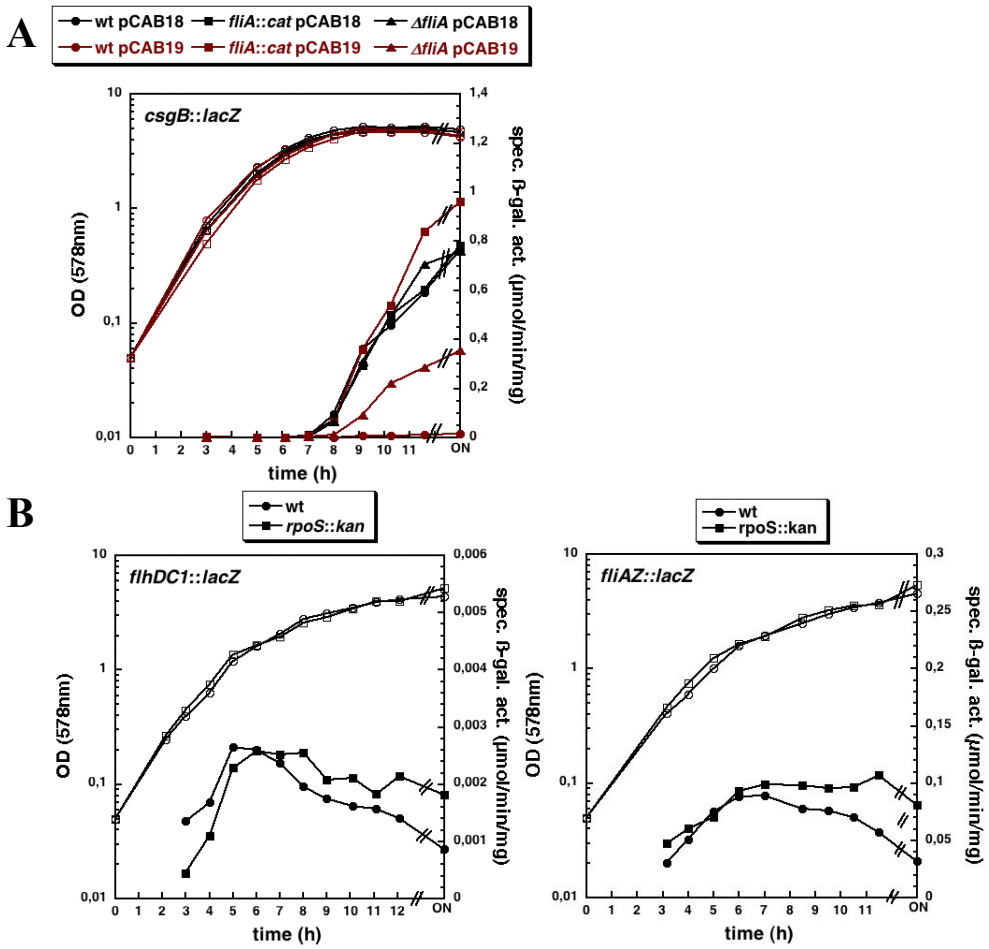

Fig. 4.6: The cellular sigma factor balance contributes to inverse regulation of motility and curli fimbriae expression. **(A)** Expression of a single copy chromosomal *csgB*::*lacZ* fusion in MC4100 wild-type (wt), polar and non-polar *fliA* mutant derivatives that carry the empty low copy plasmid pCAB18 or its derivative pCAB19, expressing the *flhDC* operon under p_{tac} promoter control; **(B)** Expression of single copy chromosomal *lacZ* fusions reflecting *flhDC* transcription and class II promoter-driven *fliZ* transcription in W3110 wild-type (wt) and *rpoS::kan* mutant derivatives. Cells were grown at 28°C in LB medium. Cultures in A were supplemented with ampicillin and 10 µM IPTG. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in the overnight cultures (ON).

Conversely, σ^S seems to play a role in the shut-down of flagellar gene expression. In wild-type cells, expression of single copy *lacZ* reporter fusions mirroring *flhDC* (class I) transcription and class II promoter-driven transcription of *fliZ* peaked in post-exponential phase (with slightly later induction of *fliZ::lacZ* expression reflecting the successive induction of class I and II genes) and declined upon entry into stationary phase (Fig. 4.6B). In contrast, expression of both fusions continued in stationary phase in a *rpoS* (σ^S) mutant background (Fig. 4.6B), corroborating similar findings obtained by other members of the Hengge group before (Pesavento et al. 2008). Thus, σ^S induction in stationary phase seems to directly or indirectly contribute to down-regulation of the flagellar gene regulon. This may again be due to increased competition between σ^S and the two sigma factors controlling flagellar gene expression (σ^{70} and σ^{28}) for RNAP core enzyme.

Together, these results demonstrate that growth phase-dependent changes in the balance between the sigma factors involved in regulation of flagella and curli fimbriae expression contribute to establishing mutual exclusion of motility and adhesion.

4.1.4 Regulation of FliZ expression and activity

Participation of σ^S in down-regulation of the flagellar gene regulon in stationary phase suggested that σ^S might be involved in inactivation of its own antagonist FliZ. The down-regulation of FliZ in stationary phase seems to be a necessary prerequisite for successful induction of curli fimbriae synthesis and was therefore analyzed in more detail.

4.1.4.1 Expression of FliZ is strongly down-regulated in stationary phase

The general expression pattern observed with the transcriptional *fliZ::lacZ* fusion in figure 4.6B, was confirmed by Northern blot analysis using a probe against *fliZ* mRNA (Fig. 4.7A). Down-regulation of *fliZ* expression upon entry into stationary phase appeared even more dramatic when this more direct method for monitoring transcription levels was used. Furthermore, two different transcripts with similar transcription patterns were detected, one corresponding in length to a transcript containing the mRNA of the entire *fliAZY* operon and a more abundant transcript that most likely contains only the *fliA* and *fliZ* mRNAs. Consistent with the transcription pattern, FliZ protein levels also raised during exponential growth, peaked in post-exponential phase and declined in stationary phase, with virtually no FliZ present in cells grown overnight (Fig. 4.7B).

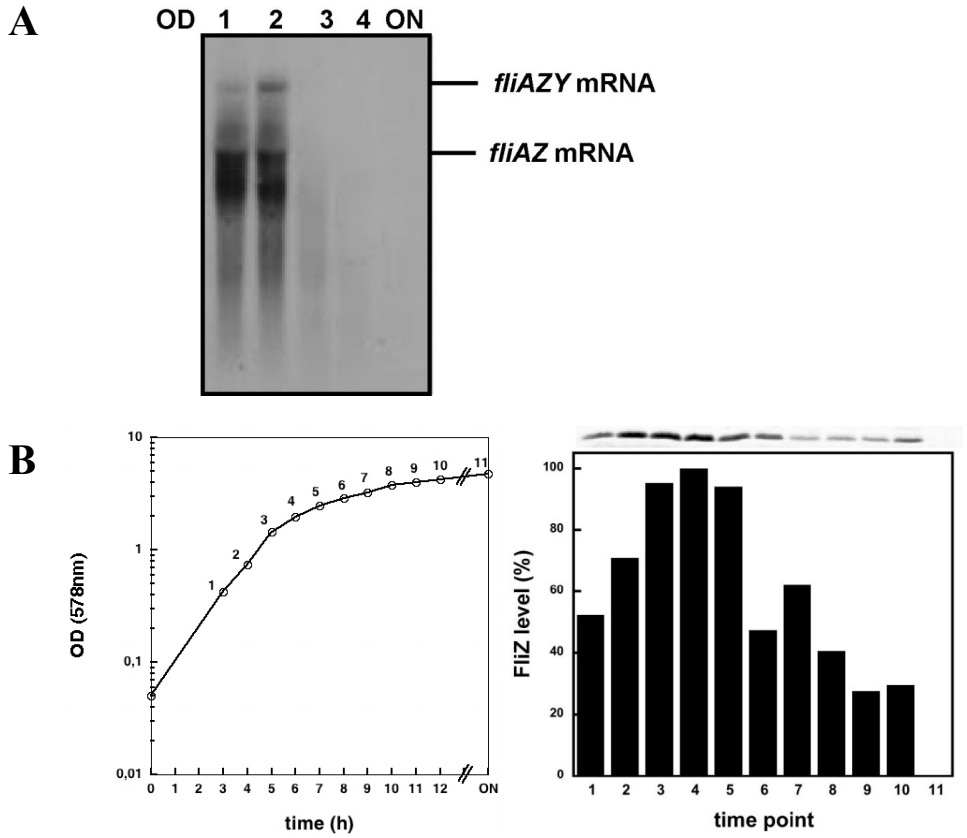

Fig. 4.7: FliZ expression is strongly down-regulated in stationary phase. (A) W3110 cells were grown in LB medium at 28°C and samples were taken at an OD_{578nm} of 1,2, 3,4 and from the overnight culture (ON). Transcripts containing the *fliZ* mRNA were detected by Northern blot analysis using a Digoxigenin-labelled probe. Transcripts were identified as *fliAZY* and *fliAZ* transcripts by comparison with a RNA size marker (not shown). **(B)** W3110 cells were grown in LB at 28°C, OD_{578nm} was measured and samples were taken along the growth curve and from the overnight culture (ON) at time points indicated in the left panel. FliZ levels were determined using a polyclonal antiserum raised against purified FliZ protein (right panel). Densitometric quantification is shown below the blot.

4.1.4.2 Search for the regulator responsible for down-regulation of FliZ expression

As the above results showed that down-regulation of *fliZ* expression in stationary phase is dramatic, it may not be solely based on increased sigma factor competition between σ^S and σ^{70}/ σ^{28}. Therefore, the participation of other regulators in down-regulation of FliZ expression in stationary phase was examined.

A recent study had identified a promoter in the intergenic region between *fliZ* and *fliY* that strongly resembles the consensus sequence of σ^S-dependent promoters and was proposed to drive $FlhD_4C_2$-independent expression of *fliY* (Zaslaver et al. 2006). FliY is a periplasmic L-cystine binding protein (Butler et al. 1993) that has been suggested to modify motility gene expression in response to binding to an extracellular factor (Mytelka and Chamberlin 1996). Northern blot analysis using a probe against *fliY* mRNA confirmed the presence of a transcript

corresponding in length to the *fliY* mRNA only (Fig. 4.8A). In contrast to the *fliAZY* and *fliAZ* transcripts, the *fliY* transcript was still present in stationary phase cells at an OD_{578} of 4 (compare figures 4.7A and 4.8A, which show the same Northern blot probed against *fliZ* and *fliY* mRNA, respectively). Considering the expression profile of *fliY* and the fact that it is partially co-transcribed with *fliZ*, FliY seemed to be a possible candidate for mediating stationary phase-induced and potentially σ^S-dependent down-regulation of FliZ. However, FliZ protein levels were unaltered in strains mutated in *fliY* (Fig. 4.8B) or expressing *fliY* from a low copy plasmid (Fig. 4.8C). Thus, FliY does not have an inhibitory influence on FliZ and is not involved in down-regulation of FliZ in stationary phase.

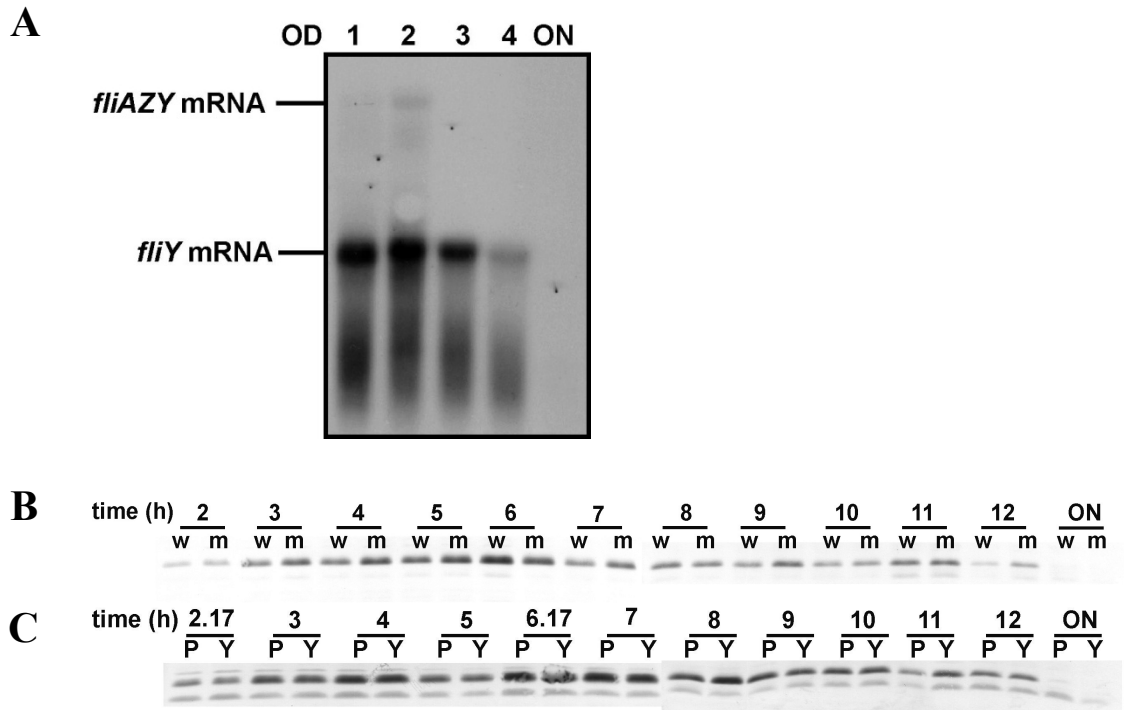

Fig. 4.8: FliY does not participate in down-regulation of FliZ. (A) Northern blot analysis of *fliY* mRNA levels. W3110 cells were grown in LB medium at 28°C and samples were taken at an OD_{578nm} of 1, 2, 3,4 and from the overnight culture (ON). Transcripts containing the *fliY* mRNA were detected by Northern blot analysis using a Digoxigenin-labelled probe. Transcripts were identified as *fliAZY* and *fliY* transcripts by comparison with a RNA length marker (not shown). The blot is identical to the Northern blot shown in figure 4.7A. Detection with the probe against *fliY* mRNA was performed after stripping off the anti-*fliZ* probe. **(B & C)** Immunoblot analysis of FliZ levels in (B) W3110 wild-type (w) and *fliY::kan* mutant (m) derivatives and (C) W3110 cells carrying either the empty low copy plasmid pCAB18 (P) or its derivatives pFliY (Y) expressing *fliY* under p_{tac} promoter control in the absence of the inducer IPTG. Cells were grown at 28°C in LB medium with (C) or without (B) ampicillin. Samples were taken along the growth curve at indicated hours after inoculation to a start OD_{578nm} of 0.05 and from the overnight culture (ON) with cultures directly compared showing a similar OD_{578nm} at the respective time points (not shown). Note that the upper one of the two visible protein bands in (B) and (C) represents the FliZ protein, as the lower band was also detected by the anti-FliZ antibody in samples taken from *fliZ* mutants (data not shown).

As down-regulation of FliZ in stationary phase was already apparent at the mRNA level (Fig. 4.7A), a small RNA-based mechanism was considered as another possibility. An increasing number of small non-coding regulatory RNAs has been identified in recent years and many of them were shown to regulate gene expression through base-pairing with target mRNAs resulting in changes in mRNA stability and translation (Gottesman 2005; Papenfort and Vogel 2009). Amongst these, the small non-coding RNA RyeB appeared to be a possible candidate for mediating down-regulation of FliZ in stationary phase, because RyeB had been identified as a stationary phase induced small RNA of unknown function (Wassarman et al. 2001; Vogel et al. 2003). A possible contribution of RyeB to down-regulation of FliZ was tested by analyzing the effects of RyeB over-expression from a high copy plasmid on *fliZ* mRNA and FliZ protein levels (Fig. 4.9). Although a slight decrease in both mRNA and protein levels could be detected in cells over-expressing RyeB, this effect is unlikely to be based on a mechanism involving direct interaction between RyeB and *fliZ* mRNA. In the case of direct interaction, the strong over-expression of RyeB should result in a much more dramatic down-regulation of *fliZ* mRNA. The observed mild down-regulation of *fliZ* mRNA and FliZ protein levels might therefore be the consequence of an indirect and most likely unphysiological effect. The RNA chaperone Hfq binds to mRNAs and small RNAs, stabilizes the latter, promotes their interaction with mRNA targets and also interacts with RNaseE, which is involved in mediating degradation of small RNA-target complexes (Aiba 2007). Therefore, over-expression of RyeB might, for example, titrate Hfq from other small RNA-based processes that may be involved in regulation of FliZ expression. Thus, small RNA-based regulation of FliZ expression cannot be ruled out, but RyeB is not the sought-after regulator mediating down-regulation of FliZ in stationary phase.

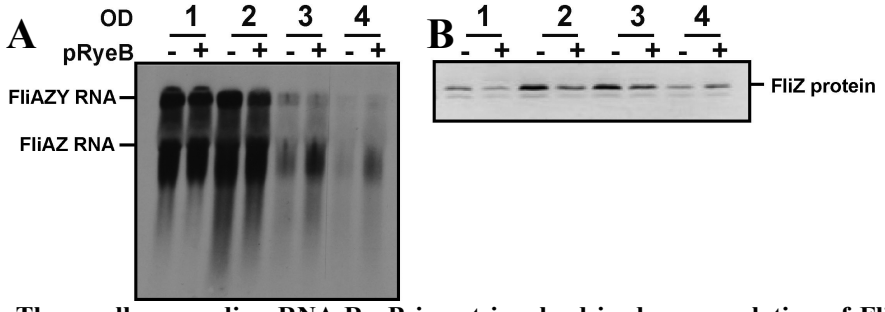

Fig. 4.9: The small non-coding RNA RyeB is not involved in down-regulation of FliZ. (A) Northern blot analysis of *fliZ* mRNA and **(B)** immunoblot analysis of FliZ protein levels in W3110 derivatives carrying the control vector pJV300 (-) or plasmid pRyeB (+), from which the small non-coding RNA RyeB is continuously expressed. Cells were grown in LB medium supplemented with ampicillin at 28°C and samples were taken at an OD_{578nm} of 1, 2, 3 and 4. *fliAZY* and *fliAZ* transcripts were identified by comparison with an RNA size marker (not shown). Note that the upper one of the two visible protein bands in (B) represents the FliZ protein, as the lower band was also detected by the anti-FliZ antibody in samples taken from *fliZ* mutants (data not shown).

Lastly, the DNA-binding repressor NsrR was analyzed for a potential participation in down-regulation of FliZ. NsrR regulates the expression of genes involved in detoxification of nitric-oxide (Bodenmiller and Spiro 2006). Recently, NsrR has been shown to also repress genes involved in motility, by binding to the promoters of flagellar genes including the *fliAZY* operon, while it positively affects adhesion through an unknown mechanism (Partridge et al. 2009). Since the *nsrR* gene (formerly known as *yjeB*) was also shown to be part of the σ^S regulon (Weber et al. 2005) this regulator seemed to be a *bona fide* candidate for mediating down-regulation of FliZ in stationary phase, which might establish an additional regulatory link between the σ^S-controlled curli cascade and flagellar gene expression.

σ^S dependence of *nsrR* expression was confirmed, using a chromosomal *nsrR::lacZ* fusion (Fig. 4.10A).

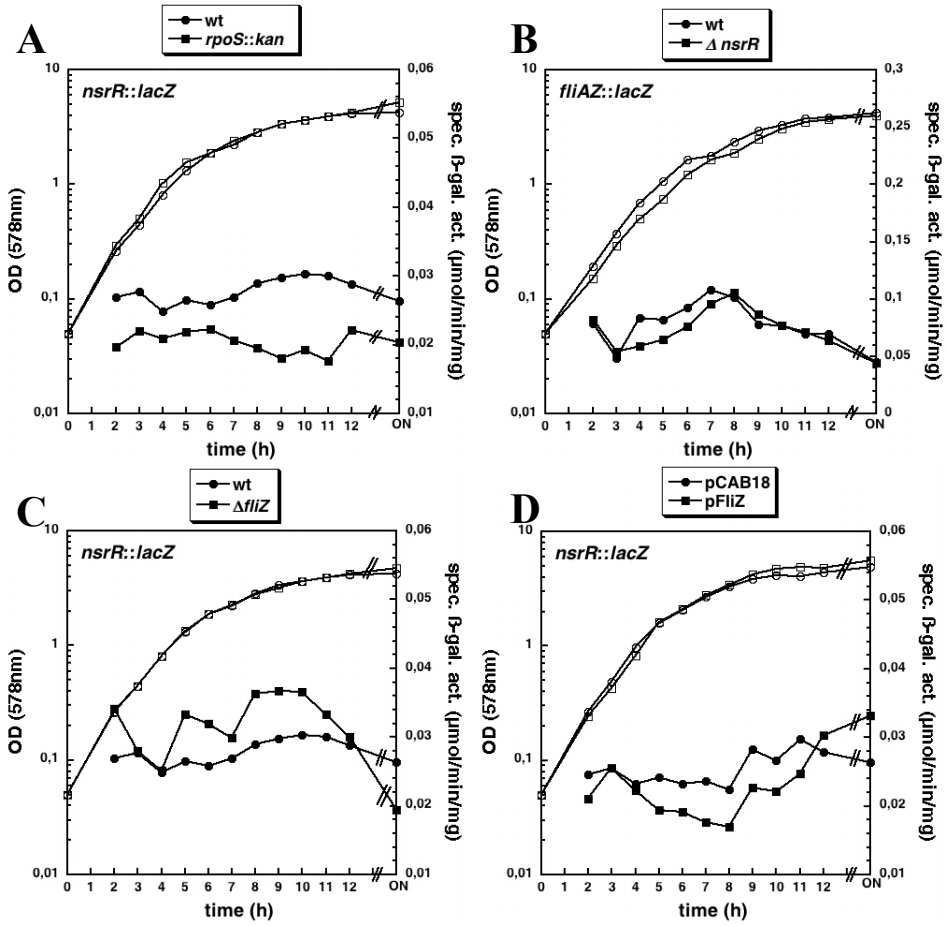

Fig. 4.10: The DNA-binding repressor NsrR is not involved in down-regulation of FliZ, but the *nsrR* gene is a FliZ target. Expression of single copy chromosomal *lacZ* fusions to **(A,C,D)** *nsrR* and **(B)** *fliZ* in W3110 wild-type cells (wt) and cells carrying the indicated mutations (A-C), or carrying either the low copy plasmid pCAB18 or its derivative pFliZ, expressing *fliZ* under p_{tac} promoter control (D). Cells were grown in LB medium at 28°C, cultures in (D) were supplemented with ampicillin. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

However, negative regulation of *fliZ* expression by NsrR could not be observed (Fig. 4.10B). The discrepancy between these results and the results published by Partridge et al. (2009) might be due to differences in the growth conditions used. Yet, the results demonstrate that NsrR is not involved in down-regulation of FliZ under conditions promoting curli fimbriae expression.

Since the *nsrR* gene is activated by σ^S it was also a potential target for regulation by FliZ. This was confirmed by experiments shown in figures 4.10C and D, which demonstrated that expression of a *nsrR::lacZ* fusion was elevated in the Δ*fliZ* mutant and repressed in the presence of *fliZ* expression from the low copy plasmid, thus adding another gene to the FliZ regulon.

4.1.5 ClpP-mediated proteolysis is required for switching from motility to curli fimbriae expression and affects the DGC/PDE balance

The above analyses failed to identify the regulator mediating the strong down-regulation of FliZ expression. However, even if the identity of an involved regulator is to be revealed in future experiments, down-regulation of FliZ expression may not be sufficient to allow for induction of curli fimbriae expression because the few remaining cell divisions that take place until cultures stop dividing in stationary phase will only lead to a slight dilution of the already synthesized FliZ protein. Yet, strong down-regulation is also observed at the protein level (Fig. 4.7B) and continues in stationary phase until FliZ is completely eliminated in cultures grown overnight, indicating that FliZ levels are further diminished even in the absence of further cell division. This suggested the presence of additional mechanisms destabilizing FliZ in stationary phase. As a first test, if proteolysis plays a role in down-regulation of FliZ, expression of a *csgB::lacZ* fusion was monitored in a strain mutated in *clpP*. ClpP is a major protease in *E. coli* that, when associated with the chaperones ClpA or ClpX, specifically recognizes, unfolds and degrades substrate proteins (Baker and Sauer 2006). A *clpP* mutation completely abolished *csgB::lacZ* expression (Fig. 4.11), indicating accumulation of a ClpP-substrate that interferes with curli fimbriae induction. This was particularly striking, since this negative influence apparently overrides the expected positive effect of accumulation of the ClpXP substrate σ^S on curli fimbriae expression. However, since the curli-negative phenotype of the *clpP* mutant was not suppressed by additional insertion of a *fliZ* mutation (Fig. 4.11),

FliZ does not seem to be responsible for repression of curli fimbriae expression in the *clpP* mutant background.

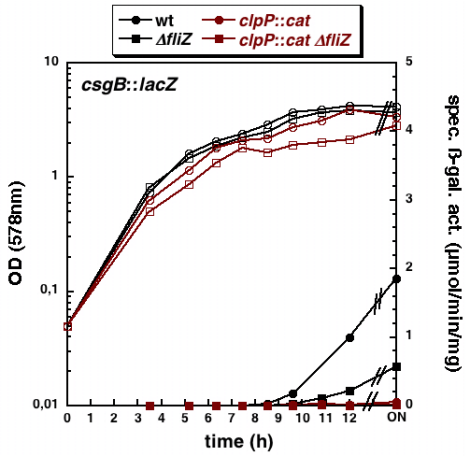

Fig. 4.11: A *clpP* mutant is unable to synthesize curli fimbriae, but FliZ is not responsible for this phenotype. Expression of a single copy chromosomal *csgB::lacZ* fusion in W3110 wild-type cells (wt) and derivatives carrying the indicated mutations. Cells were grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

Failure of the *fliZ* mutation to suppress the curli-negative phenotype of the *clpP* mutant does not, however, rule out that FliZ is regulated by ClpP-mediated proteolysis, since accumulation of several factors might collectively and redundantly contribute to repression of curli fimbriae synthesis in the *clpP* mutant. Due to the presence of multiple proteolysis substrates in both the curli cascade (e.g. the ClpXP substrate σ^S) and the flagellar gene regulon (e.g. the Lon substrate σ^{28}), mutations in the protease genes have multifaceted effects on the expression of members of both cascades, e.g. through shifts in sigma-factor competition. Therefore, FliZ was ectopically expressed from the low copy vector pCAB18 to uncouple its expression from these indirect effects in order to analyse the influence of a mutation in *clpP* on FliZ protein levels. Unexpectedly, a mutation in *clpP* strongly reduced FliZ levels when the protein was expressed under p_{tac} promoter control, and this effect was most pronounced during post-exponential growth and in stationary phase (Fig. 4.12A). Down-regulation of FliZ in the *clpP* mutant could be suppressed by a mutation in *rpoS*, indicating that strong accumulation of σ^S in the *clpP* mutant is responsible for this down-regulation (Fig. 4.12A). It has been reported before that p_{tac}-promoter-driven expression is down-regulated in strains defective in *clpP*. This is due to sigma factor competition, which results in inhibition of σ^{70}-controlled expression from the p_{tac}-promoter by high levels of σ^S that accumulate in the *clpP* mutant (Schweder et al. 2002). Consistently, Northern blot analysis of *fliZ*-mRNA levels demonstrated that the influences of ClpP and σ^S on FliZ also manifest themselves at the

mRNA level, indicating that the observed regulation indeed reflects the effects that these regulators exert on p_{tac}-promoter-driven expression (Fig. 4.12B). Moreover, sensitivity of p_{tac}-promoter-driven expression to alterations in the sigma factor balance may also be responsible for the growth phase-dependent changes in FliZ levels, which result in an expression pattern very similar to the one observed with *fliZ* being expressed under the control of its native promoter.

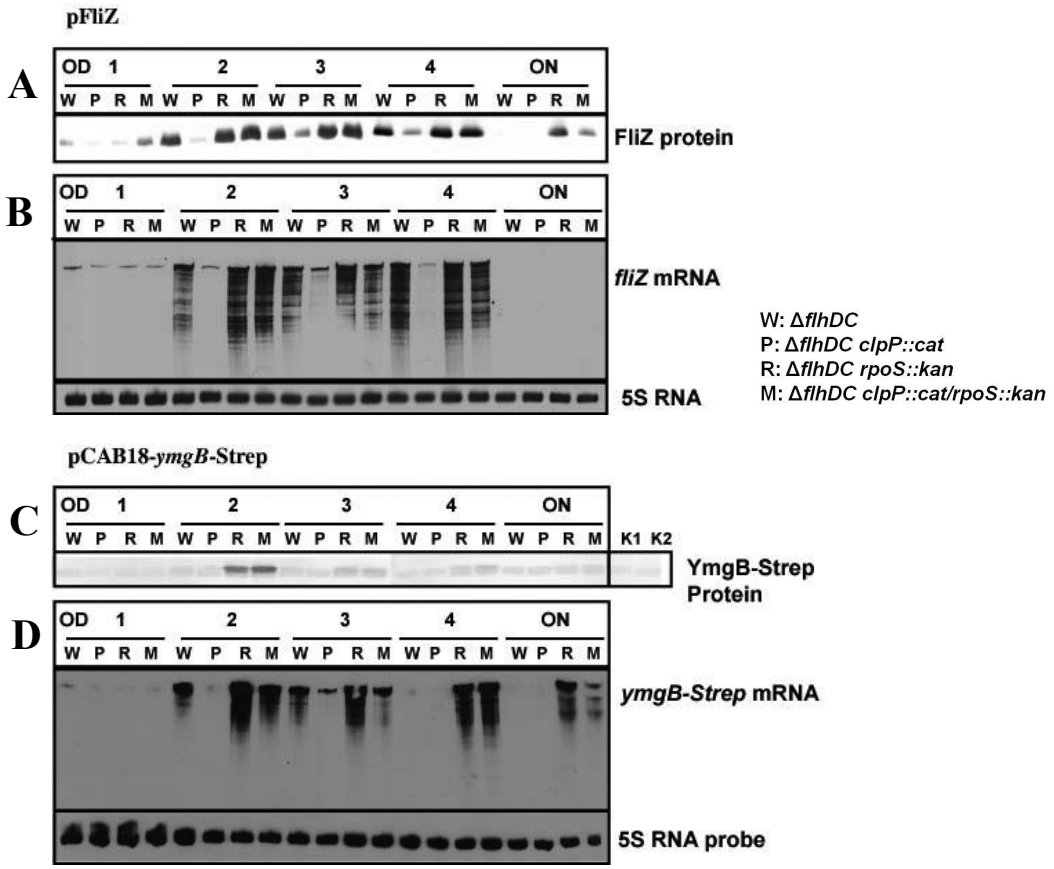

Fig. 4.12: p_{tac}-promoter driven expression of *fliZ* and *ymgB* from the low copy number plasmid pCAB18 shows growth phase-dependent variations and is subject to regulation by σ^S and ClpP. W3110 cells carrying the indicated mutations and expressing either *fliZ* **(A&B)** or *ymgB-Strep* (encoding a Strep-tagged version of the YmgB protein) **(C&D)** under p_{tac}-promoter control from the low copy plasmid pCAB18 were grown in LB medium supplemented with ampicillin at 28°C without inducer. Samples were taken at an OD_{578nm} of 1, 2, 3, 4 and from overnight cultures (ON). FliZ and YmgB levels were determined by immunoblot analysis using polyclonal antisera against FliZ (A) or against the Strep-Tag (C), respectively. Transcripts containing *fliZ* mRNA (B) and *ymgB* mRNA (D) were detected by Northern blot analysis using Digoxigenin-labelled probes. As a control shown underneath the respective Northern blot, both blots were also probed against 5S-RNA, the levels of which should not be affected by any of the mutations. K1 and K2 indicate control samples taken from overnight cultures of W3110 Δ*flhDC* and W3110 Δ*flhDC clpP::cat* cells, respectively, that carry the empty vector pCAB18. As the lower one of the two protein bands visible in other samples shown in C is also detected in these samples, the YmgB-Strep protein is represented by the upper band. Note that the Δ*flhDC* mutation was introduced into all strains to avoid the growth defects caused by introduction of a *clpP* mutation into a strain normally expressing the flagellar gene regulon.

The notion that the observed effects reflect regulation of the p_{tac}-promoter rather than indicating post-transcriptional regulation of FliZ was further confirmed by the finding that

single and double mutations in *clpP* and *rpoS* had the same effect on the levels of another protein (YmgB, a small protein that modulates the activity of the Rcs system (Tschowri et al. 2008)) ectopically expressed from pCAB18 under the same conditions (Fig. 4.12C). Again, regulation could also be observed at the mRNA level (Fig. 4.12D). Thus, p_{tac}-promoter-driven expression of FliZ could not be used to analyse the role of ClpP (and σ^S) in post-transcriptional/post-translational regulation of FliZ. Due to the difficulties associated with the *in vivo* analysis of potential FliZ proteolysis, future experiments will use *in vitro* proteolysis systems established in the Hengge group to clarify if FliZ is subject to proteolysis.

If FliZ is not responsible for the curli-negative phenotype of the *clpP* mutant, what causes curli repression in this strain? In contrast to the *fliZ* mutation, a mutation in the flagellar master regulator operon *flhDC* completely suppressed curli repression in the *clpP* mutant (Fig. 4.13A), indicating that the inhibitor is part of the flagellar gene regulon.

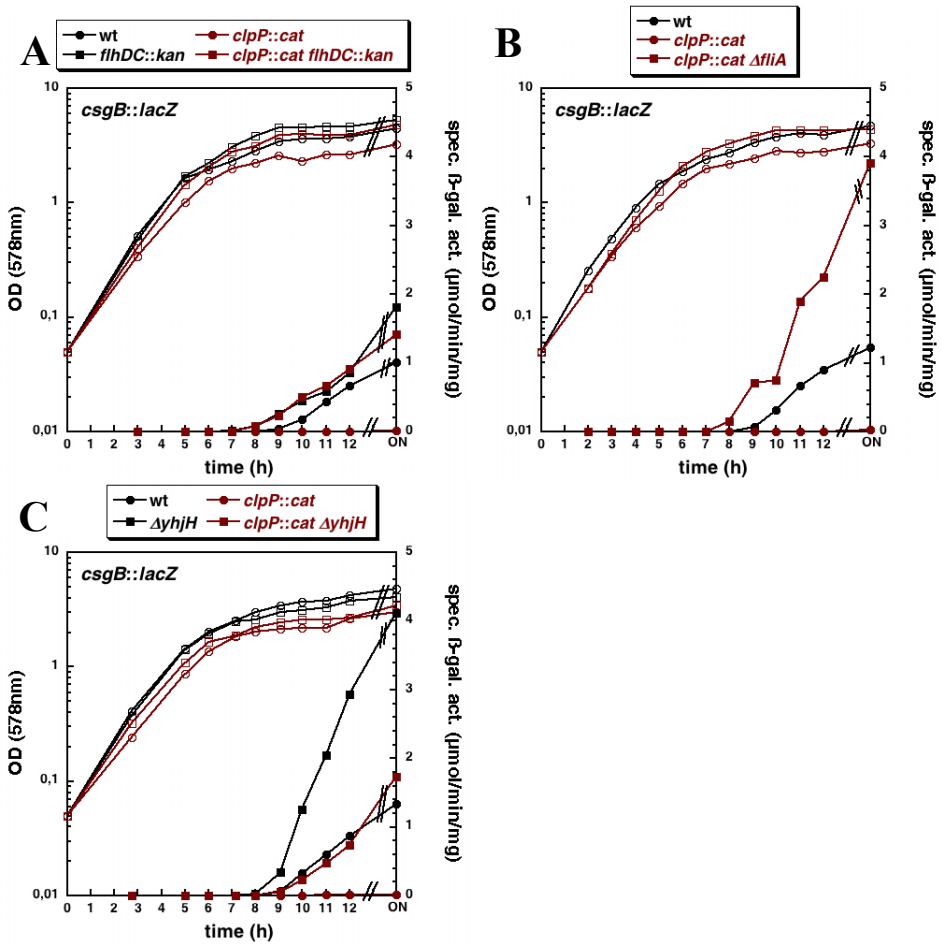

Fig. 4.13: A mutation in the flagellar class III gene *yhjH* suppresses the curli-negative phenotype of the *clpP* mutant. Expression of a single copy chromosomal *csgB::lacZ* fusion in W3110 wild-type cells (wt) and derivatives carrying the indicated mutations. Cells were grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

Further analyses of flagellar gene mutants showed that both polar and non-polar mutations in *fliA* (encoding σ^{28}) and a mutation in the class III gene *yhjH* were able to restore curli fimbriae expression in the *clpP* mutant (Fig. 4.11B/C).

yhjH encodes an EAL protein and phosphodiesterase that degrades the c-di-GMP, which otherwise interferes with motility by binding to the effector protein YcgR (Ko and Park 2000b; Rychlik et al. 2002; Frye et al. 2006; Ryjenkov et al. 2006). Studies performed by the Hengge group in parallel to this work had identified the σ^S-dependent GGDEF proteins YegE and YedQ as factors responsible for the synthesis of c-di-GMP degraded by YhjH (Pesavento et al. 2008). This c-di-GMP control module essentially contributes to throwing the switch from motility to adhesion, since σ^S-mediated induction of c-di-GMP synthesis by YegE and YedQ finally outbalances YhjH-mediated c-di-GMP hydrolysis. This happens as a result of σ^S induction (and consequently YegE and YedQ induction) and concomitant cessation of flagellar gene expression (and thereby YhjH expression) during entry into stationary phase. The resulting elevated c-di-GMP level leads to inhibition of motility via YcgR. At the same time it contributes to the c-di-GMP-mediated induction of curli fimbriae expression through activation of the curli regulator gene *csgD*, thereby connecting inhibition of motility to induction of curli fimbriae synthesis (shown by Gisela Becker in (Pesavento et al. 2008)).

Suppression of the curli-negative phenotype of the *clpP* mutant by mutation of *yhjH* suggested that the *clpP* mutant is unable to sufficiently overcome the negative effect of YhjH on curli fimbriae expression. Two scenarios causing insufficient down-regulation of YhjH in the *clpP* mutant could be envisaged: Firstly, YhjH itself may be a substrate of ClpP-mediated degradation. Alternatively, prolonged presence of $FlhD_4C_2$, which has been shown to be subject to ClpXP-mediated degradation (Tomoyasu et al. 2003) may result in prolonged expression of flagellar class II and III genes, including *yhjH*. This may prevent sufficient down-regulation of *yhjH* expression to allow for induction of curli fimbriae expression. Experiments performed by other members of the Hengge group to test the first hypothesis demonstrated that YhjH is unlikely to be subject to proteolysis (Pesavento et al. 2008). Moreover, expression of a *yhjH::lacZ* fusion was found to be substantially prolonged in the *clpP* mutant (Fig. 4.14, this experiment was performed by Alexandra Possling), supporting the hypothesis that the curli-negative-phenotype results from insufficient down-regulation of *yhjH* expression. Together these results strongly suggest that proteolysis of $FlhD_4C_2$ is required to efficiently and precisely shut down flagellar gene expression around an OD_{578nm} of 2.5. This is a prerequisite for the YegE/YedQ-induced shift to higher c-di-GMP levels, which results in induction of curli fimbriae expression and shut down of motility.

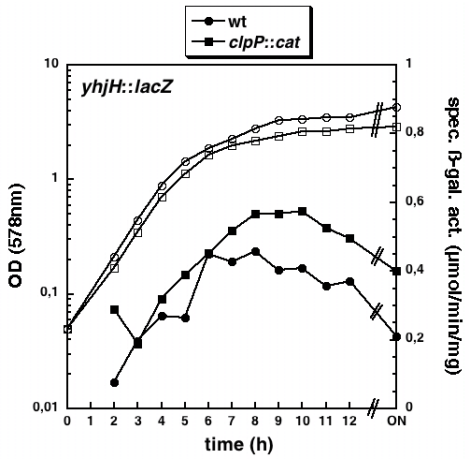

Fig. 4.14: The ClpP protease plays an essential role in down-regulation of *yhjH* expression. Expression of a single copy chromosomal *yhjH::lacZ* fusion was measured in W3110 wild-type (wt) and *clpP::cat* mutant derivatives. Cells were grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON). This experiment was performed by Alexandra Possling.

In summary, the results shown in the first part of this chapter identified FliZ as a regulator under flagellar control that interferes with curli fimbriae expression and the general stress response by repressing σ^S-dependent gene expression for as long as flagellar gene expression continues. Moreover, FliZ-mediated regulation is integrated with several other layers of inverse control of motility and curli fimbriae expression including c-di-GMP-mediated control and sigma factor competition. In this complex network, the growth phase-dependent induction and precise shut-down of components of the two regulatory cascades was shown to play an essential role. Regulators possibly involved in down-regulation of FliZ in stationary phase, however, remained elusive. In the next part, results elucidating the mechanism by which FliZ interferes with σ^S-dependent gene expression will be presented.

4.2 The mechanism of FliZ action

Before the work for this thesis had commenced, several groups had analyzed FliZ-dependent effects on motility in *E. coli* (Mytelka and Chamberlin 1996) and *Salmonella* (Ikebe et al. 1999; Kutsukake et al. 1999) and on the expression of *Salmonella* pathogenicity island 1 (SPI1) genes (Lucas and Lee 2001) that code for a type III secretion system, several regulators and secreted effectors essential for invasion of the host´s intestinal epithelium (Lostroh and Lee 2001). However, details on the regulatory mechanisms responsible for these effects had not been identified in any case. To elucidate the mechanism by which *fliZ* exerts its effects in *E. coli*, comprehensive *in vitro* and *in vivo* analyses were performed, which will be presented next.

4.2.1 FliZ acts through a novel mechanism

4.2.1.1 In *E. coli* FliZ does not act through the homolog of the *Salmonella* pathogenicity island 1 regulator HilA

The finding that in *Salmonella*, FliZ mediates its effect on SPI1 expression via an uncharacterized influence on the transcriptional regulator HilA (Lucas et al. 2000; Iyoda et al. 2001; Lucas and Lee 2001) represented the only detail on a regulatory mechanism involving FliZ known before this work had started. The *E. coli* gene *ygeH*, which is located in a cryptic gene cluster comprising remnants of genes from several pathogenicity islands, is a homolog of the *Salmonella hilA* gene (Ren et al. 2004). In order to exclude that the observed FliZ-mediated effects on σ^S-dependent gene expression are mediated by YgeH, a *ygeH* mutation was constructed to test if it is able to alleviate curli repression by ectopic expression of *flhDC* and *fliZ* from the low copy vector pCAB18. As shown in figure 4.15, introduction of the *ygeH* mutation did not affect repression of *csgB::lacZ* expression, indicating that the HilA homolog YgeH does not play a role in FliZ-mediated repression of curli fimbriae expression.

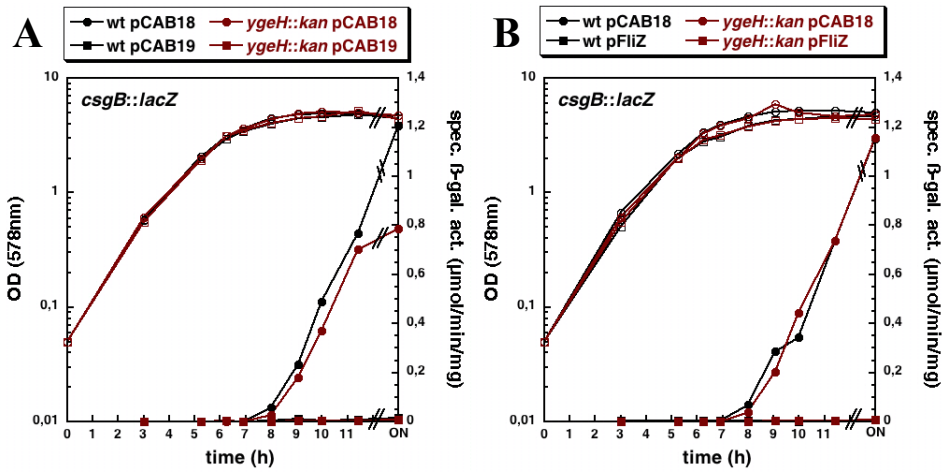

Fig. 4.15: FliZ does not require YgeH to repress curli fimbriae expression. Expression of a single copy chromosomal *csgB*::*lacZ* fusions in MC4100 wild-type (wt) and *ygeH::kan* mutant cells carrying either the empty low copy plasmid pCAB18 or its derivatives pCAB19 **(A)** or pFliZ **(B)** expressing *flhDC* or *fliZ*, respectively, under p_{tac} promoter control in the presence (A) and absence (B) of IPTG. Cells were grown at 28°C in LB medium supplemented with ampicillin. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

4.2.1.2 FliZ does not interact with σ^S

Considering its general effect on the σ^S regulon, FliZ seemed to be a *bona fide* candidate for an anti-sigma factor that interferes with σ^S activity through direct interaction. Alternatively, FliZ may interfere with $E\sigma^S$ activity by directly inactivating σ^S-controlled promoters. In order to distinguish between these two possibilities, it was first tested if FliZ directly interacts with σ^S. To this end, a bacterial two-hybrid system was used, which represents an efficient method to detect protein-protein interaction *in vivo*. In this system one protein (bait protein, expressed from vector pBT) is fused to the full-length bacteriophage λ repressor. The potential interaction partner (target protein, expressed from vector pTRG) is fused to the N-terminal domain of the α subunit of RNA polymerase. If the two proteins interact, they induce transcription of the HIS3 reporter gene. HIS3 gene expression complements the histidine auxotrophy of the reporter strain. Strongly induced HIS3 expression in the presence of bait-target interaction allows for growth in the presence of 2-amino-1,2,4-triazole (3-AT), which is a competitive inhibitor of the His3 enzyme. Activation of the HIS3 reporter gene is based on the presence of a λ operator site upstream of the reporter gene that binds the λ repressor-bait-fusion construct. Interaction between the bait and target proteins recruits RNAP to the promoter and stabilizes RNAP binding, thereby activating transcription of the HIS3 gene. When potential interaction between σ^S and FliZ was tested using this system, no growth on selective medium containing the His3 inhibitor 3-AT could be detected with reporter strains co-transformed with plasmids pBT-RpoS (expressing σ^S fused to the λ repressor) and pTRG-

FliZ (expressing FliZ fused to RNAP α-NTD) (Fig. 4.16A), neither at 37°C, nor at 28°C. In contrast, the interaction between σ^S and its proteolysis targeting factor RssB, which was used as a positive control, was confirmed by growth of reporter cells carrying both pBT-RpoS and pTRG-RssB in the presence of 3-AT, thus validating functionality of the system. Moreover, presence of pBT-RpoS restored *csgB::lacZ* expression in a *rpoS::kan* mutant and pTRG-FliZ repressed *csgB::lacZ* expression, demonstrating that σ^S and FliZ are both still able to exert their physiological functions as fusion proteins expressed from these plasmids (Fig. 4.16B/C). In conclusion, these results suggest that FliZ does not interact with σ^S *in vivo*. Thus, it seemed unlikely that FliZ interferes with σ^S activity by acting as a typical anti-sigma factor. This was further corroborated by the observation that over-expression of FliZ is toxic, even in exponentially growing cells that contain no or very low amounts of σ^S (data not shown). This toxicity cannot be explained by a simple anti-σ^S-mechanism involving interaction and sequestration of σ^S by FliZ, as even total depletion of σ^S (in a *rpoS* mutant) does not have a similar effect on growth.

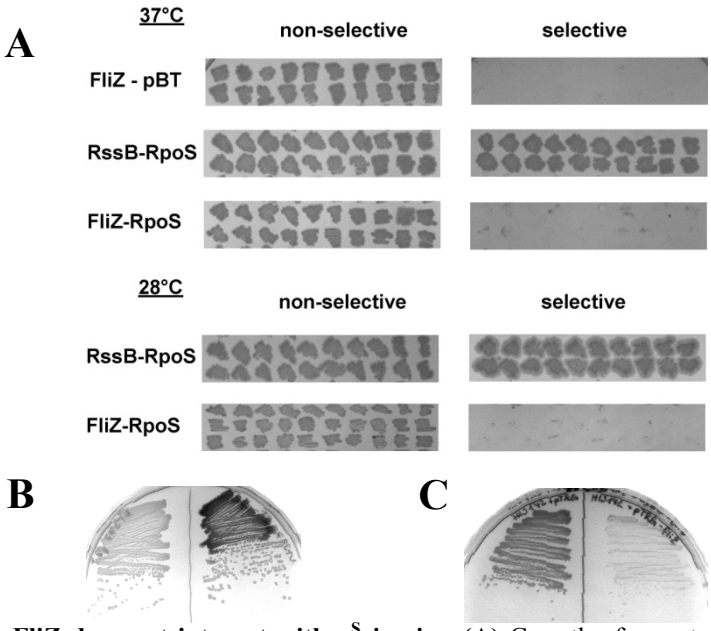

Fig. 4.16: FliZ does not interact with σ^S *in vivo*. (A) Growth of reporter cells co-transformed with a vector control and/or derivatives of the pBT and pTRG plasmids expressing fusions of the indicated proteins to the λ repressor (σ^S (RpoS)) or RNAP αNTD (FliZ, RssB), respectively, in the absence (non-selective) and presence (selective) of the His3 inhibitor 3-AT at 37°C and 28°C as indicated. For further information on experimental details refer to the Material and Methods section. **(B)** Expression of a single copy chromosomal *csgB::lacZ* fusion in a MC4100 derivative carrying a *rpoS::kan* mutation and either the pBT vector (left side of the plate) or its derivative expressing σ^S fused to the λ repressor (right side of the plate). **(C)** Expression of a single copy chromosomal *csgB::lacZ* fusion in MC4100 cells carrying either the pTRG vector (left side of the plate) or its derivative expressing FliZ fused to RNAP αNTD. Cells in C and D were grown at 28°C on LB medium plates supplemented with X-Gal for detection of *lacZ* expression (indicated by dark colour of cells) and chloramphenicol (B) or tetracylin (C).

4.2.1.3 FliZ is a novel DNA-binding regulator

The C-terminal half of FliZ contains a region with homology to the core-binding domain of the phage integrase family. This family includes various phage integrase enzymes, such as λ phage integrase and recombinase enzymes, e.g. Cre from the P1 phage and XerD from *E. coli*. The core-binding domain of phage integrase family members binds to certain elements in the DNA sequences recognized by these enzymes (Tirumalai et al. 1998; Swalla et al. 2003). Due to this partial homology to a DNA-binding domain, it seemed possible that FliZ acts by interfering with $E\sigma^S$ activity directly at target promoters.

In order to test whether FliZ is able to bind to DNA, the protein was purified and incubated with promoter DNA of the FliZ- and σ^S-dependent curli control genes *mlrA* and *yciR* in electrophoretic mobility shift assays (EMSA). FliZ bound to both promoter regions resulting in protein-DNA complexes with reduced electrophoretic mobility (Fig. 4.17 A), thus identifying FliZ as a DNA-binding regulator. In contrast, FliZ did not bind to a control fragment comprising part of the translated region of the *mlrA* gene, nor to the σ^{70}-dependent promoter of the *rpoS* gene, that encodes σ^S (Fig. 4.17B).

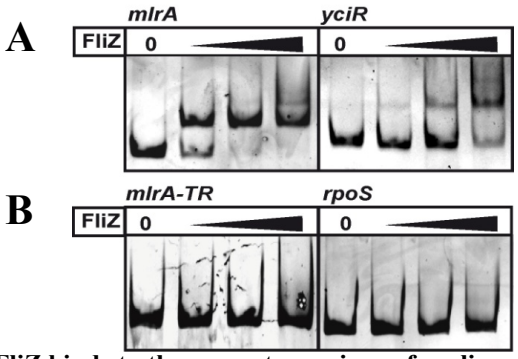

Fig. 4.17: FliZ binds to the promoter regions of curli control genes. DNA-fragments comprising the promoter regions of **(A)** the σ^S-dependent curli genes *mlrA* and *yciR* and **(B)** control fragments containing part of the translated region of the *mlrA* gene (*mlrA*-TR) and the σ^{70}-dependent *rpoS* promoter were incubated with purified FliZ protein (20, 40, 80 nM) in electrophoretic mobility shift assays.

The whole genome transcription profiling experiment presented in section 4.1.2.2 had identified a large number of FliZ-dependent genes (see Tab. 4.1). As mentioned above, most of the genes negatively regulated by FliZ are also σ^S-dependent (including the curli genes, other genes of the σ^S regulon, i.e. *gadBC*, *gadE*, *hdeAB*, *yjbJ,* and *nsrR*, which had not been identified in the microarray experiment but was demonstrated to be σ^S-controlled and FliZ-repressed in Fig. 4.10). In addition, the FliZ regulon also comprises some negatively FliZ-dependent genes that are not regulated by σ^S (e.g. *gatA*) as well as some positively FliZ-

dependent genes that are either regulated by σ^S (*chaB*, *ynhG*) or independent of σ^S (e.g. *malE*, *malK*). In order to test which of these genes are direct FliZ targets, EMSA experiments were performed with promoter DNA from selected genes of these groups. Interestingly, FliZ specifically bound to promoter DNA of genes which, like *mlrA* and *yciR*, are expressed from σ^S-dependent promoters and showed negative regulation by FliZ in the microarray experiments (Fig. 4.18A). In contrast, promoters with any of the other patterns of FliZ- and σ^S dependence were not bound (Fig. 4.18C). Thus, FliZ exerts its negative influence on σ^S-dependent gene expression by specifically binding to σ^S-controlled promoters. Other modes of regulation by FliZ seem to be indirect.

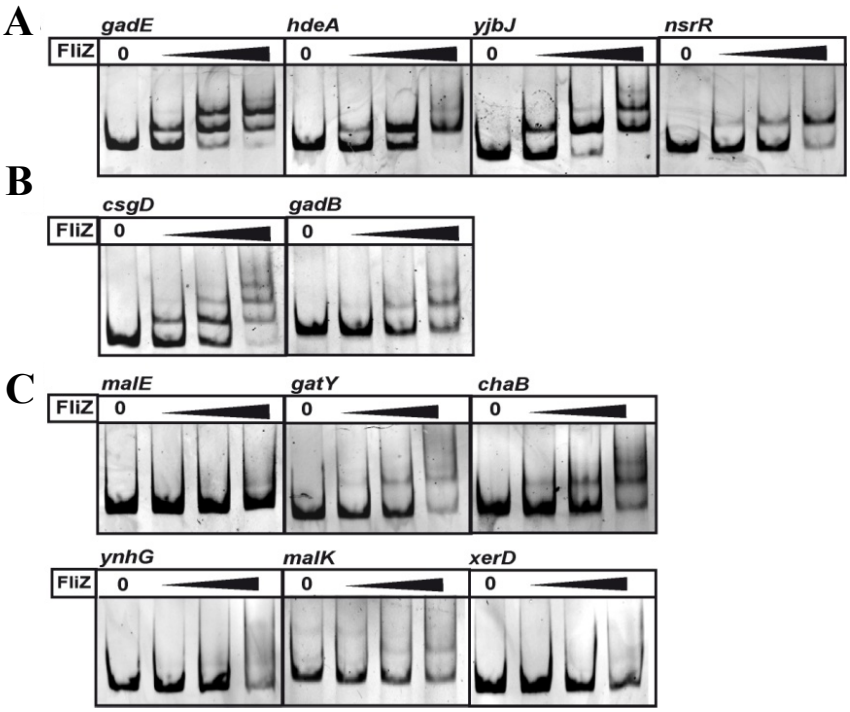

Fig. 4.18: FliZ specifically binds to σ^S-dependent promoters. DNA-fragments containing promoters of FliZ-dependent genes identified in the whole genome transcription profiling experiment (Tab. 4.1) that were either negatively FliZ-dependent and σ^S-controlled **(A&B)** or showed other patterns of FliZ- and σ^S dependence **(C)** were incubated with purified FliZ protein (20, 40, 80 nM) in electrophoretic mobility shift assays. The *nsrR* gene had not been identified in the whole genome transcription profiling experiment but was included into (A) since it was shown above to be negatively FliZ-dependent and σ^S-controlled (see Fig. 4.10). *gatY* was incuded as it is the first gene in a σ^S-independent operon containing six genes, three of which (*gatA,B,Z*) had been identified as negatively FliZ-dependent in the whole genome transcription profiling experiment.

While some promoters did not show any binding to FliZ (e.g. the *rpoS* or *malE* promoters), others bound weakly to FliZ at higher concentrations, resulting in "smearing" e.g. of the *gatY* promoter fragment rather than in increasing intensity of a clearly shifted promoter DNA fragment as e.g. with *mlrA* promoter DNA. Thus, in addition to promoters that bind FliZ with high affinity, such as the promoters shown in Fig. 4.18A, other promoters may contain

sequence features weakly resembling FliZ-binding determinants, resulting in low-affinity binding at high FliZ concentrations, which may not, however, be relevant *in vivo*. This may also be the case for the indirectly σ^S-dependent *gadB* promoter and for the σ^S-dependent *csgD* promoter (Fig. 4.18B), or for the secondary binding sites observed for *gadE* and *yjbJ*. Alternatively, weak binding of FliZ e.g. to the *csgD* promoter might be due to the absence of additional regulators, which *in vivo* bind to the same promoter region and may influence FliZ binding, resulting in lower binding affinities under the assay conditions used here.

4.2.1.4 FliZ recognizes and specifically binds to the -10 region of σ^S-dependent promoters

In order to determine the FliZ binding sites, DNaseI-footprint assays were performed with promoter regions of *mlrA*, *yciR*, *gadE* and *hdeA* (Fig. 4.19).

In the *mlrA*, *gadE* and *hdeA* promoters, the areas protected from DNaseI digestion by FliZ binding overlapped with the -10 elements and additional binding sites were identified further upstream and/or downstream. In the *yciR* promoter, FliZ bound slightly upstream of the -10 element, i.e. to the spacer region, the -35 element and further upstream. As the -10 and -35 elements are the promoter determinants recognized by sigma factors, these results suggest that FliZ binds to the same promoter elements as σ^S.

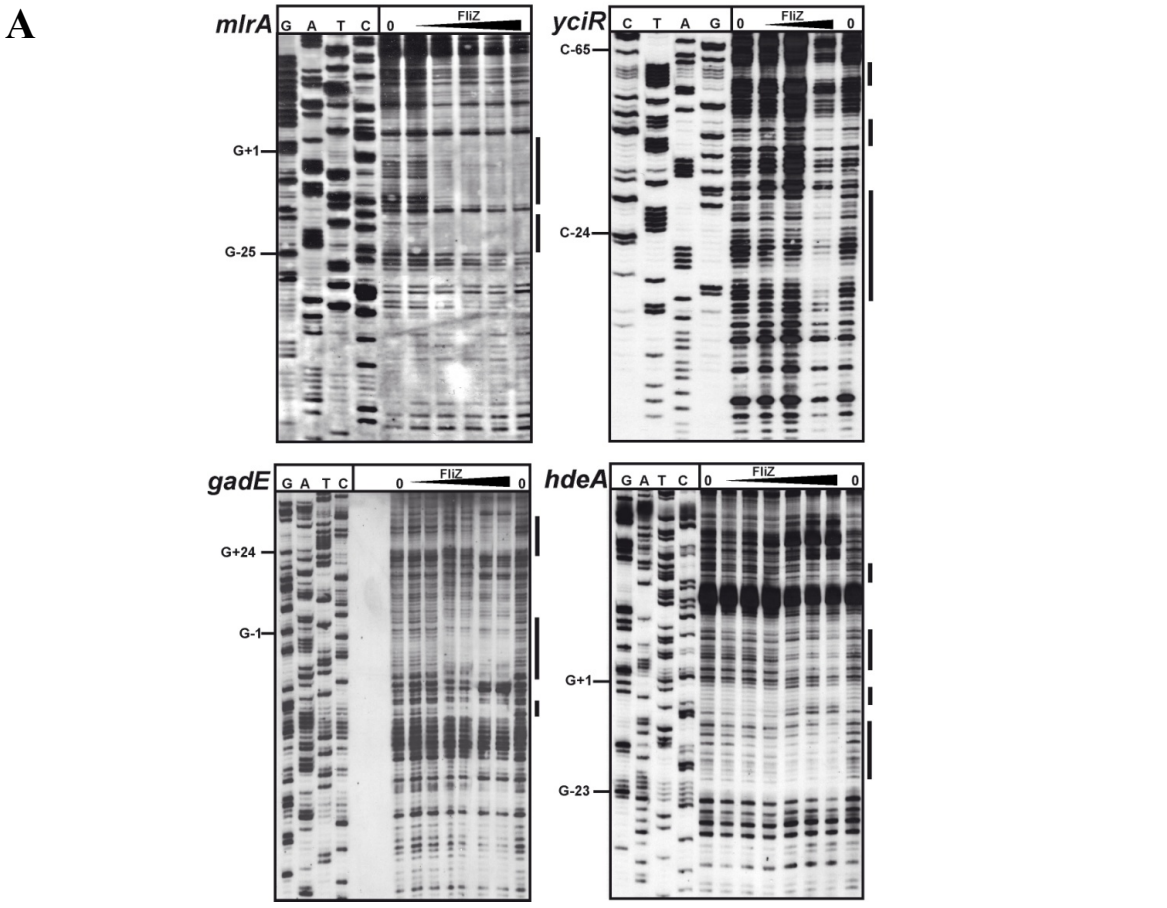

Fig. 4.19: FliZ-binding sites in σ^S-dependent promoters. (A) FliZ-binding sites were determined by non-radioactive DNaseI footprint analyses, using purified FliZ and Digoxigenin-labelled DNA fragments containing the promoter regions of the *mlrA*, *yciR*, *gadE* and *hdeA* genes. FliZ-binding sites are indicated by bars and were mapped to the promoter sequences, where they are marked by boxes **(B)**. Transcriptional start sites for all genes had been determined before: *mlrA*: (Franziska Mika, Alexandra Possling, Regine Hengge, unpublished results), *yciR*: (Cairrao et al. 2001) and also see below (Fig. 4.20), *gadE*: (Ma et al. 2004), *hdeA*: (Arnqvist et al. 1994; Itou et al. 2009). -10 and -35 elements are coloured in red, transcriptional start sites are printed as bold, red, underlined letters, a potential alternative -10 region in the *yciR* promoter (for information see below) is indicated by bold, italic letters. The sequencing reactions were performed with the same labelled primer used for the footprint experiments. Numbers indicate positions relative to the transcriptional start sites.

Since the *yciR* promoter was the only promoter tested where FliZ did not directly bind to the -10 element, primer extension experiments were performed to test whether the transcriptional start site identified by Cairrao et al. (2001) is correct. As shown in figure 4.20, the primer extension experiment identified the same 5´-mRNA end for *yciR* that has been described by Cairrao et al. (2001) and that is indicated in Fig. 4.19B.

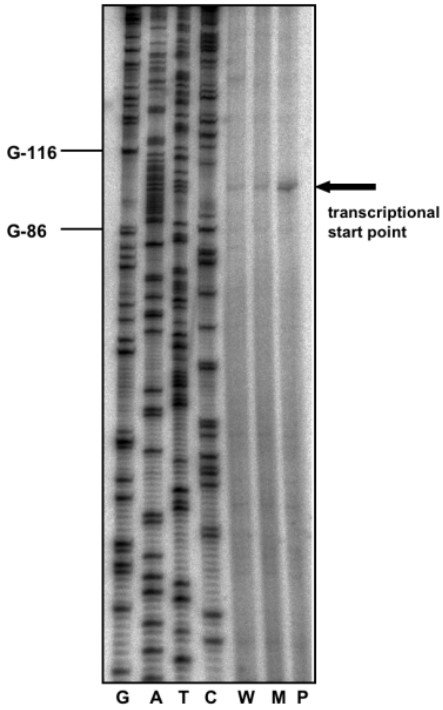

Fig. 4.20: Determination of the 5´-end of *yciR* mRNA by primer extension. W3110 wild-type cells (W) and derivatives carrying a *yciR::kan* mutation (M) or a plasmid (P) with a DNA fragment comprising the *yciR* promoter region and part of the translated region were grown in LB medium at 28°C to an OD_{578nm} of 3.5, before RNA samples were taken and primer extension was performed using a [γ-^{32}P]ATP-labelled primer. The sequencing reactions were performed with the same labelled primer. Numbers indicate positions relative to the translational start site.

However, it is possible that this site is not the actual transcriptional start site but a 5´-end generated by RNA processing. Six base pairs further upstream lies a sequence with closer resemblance to the σ^S-dependent consensus -10 promoter element than the one described by Cairrao et al. (2001) and this potential promoter sequence overlaps with the FliZ-binding site in this promoter (Fig. 4.19B). Since this putative -10 element contains a C(-13), which is known to enhance σ^S selectivity, and *yciR* expression depends on σ^S (Weber et al. 2006), this sequence is likely to constitute the actual -10 element of the *yciR* promoter. Thus, also for *yciR*, FliZ seems to bind to the extended -10 promoter region.

Within the upstream regulatory region of the *gadE* gene, several transcriptional start sites have been identified (Hommais et al. 2004; Ma et al. 2004; Weber et al. 2005). FliZ binds to the promoter located closest to the translational start site (Fig. 4.19B). Since it is not clear which, if any, of the promoters is directly σ^S-dependent (e.g. one of the identified promoters that was shown to drive σ^S-dependent, stationary-phase induced transcription of *gadE* is most likely indirectly regulated by σ^S through the σ^S-dependent regulator GadX (Weber et al. 2005)), the σ^S and FliZ dependence of this promoter was examined. A *lacZ* fusion to only this promoter showed reduced expression in a *rpoS* mutant, while post-

exponential expression levels were higher in a Δ*fliZ* mutant as compared to the wild-type (Fig. 4.21). This result confirms that the promoter described by Ma et al. (2004) is indeed active (albeit expression from this promoter is very low) and that expression from this promoter shows the typical regulatory pattern exhibited by genes directly targeted by FliZ.

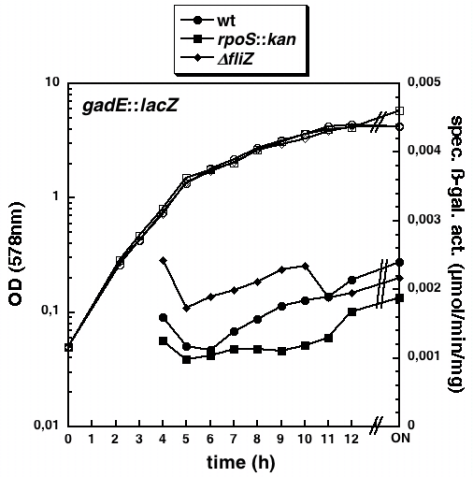

Fig. 4.21: The *gadE* promoter closest to the translational start site is active and regulated by σ^S and FliZ. Expression of a single copy chromosomal *gadE*::*lacZ* fusion exclusively reflecting expression from the promoter bound by FliZ in W3110 wild-type (wt), *rpoS::kan* and *ΔfliZ* mutant cells grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

4.2.1.5 A sequence element present in both the *mlrA* and *yciR* promoters is not essential for FliZ binding

Strikingly, the *mlrA* and *yciR* promoters both contain the identical sequence motif CAAAACTG, mainly located in the spacer region in similar distance to the transcriptional start site and in both promoters this motif is part of the site protected by FliZ binding in the DNaseI footprint experiments (Fig. 4.19). To test whether this motif plays a role in FliZ binding, mutations were introduced into residues of this motif in the *mlrA* and *yciR* promoters and the mutated promoters were tested for their ability to bind FliZ. None of the mutations affected FliZ binding to the *mlrA* and *yciR* promoters (Fig. 4.22), indicating that this sequence motif is not essential for promoter recognition by FliZ.

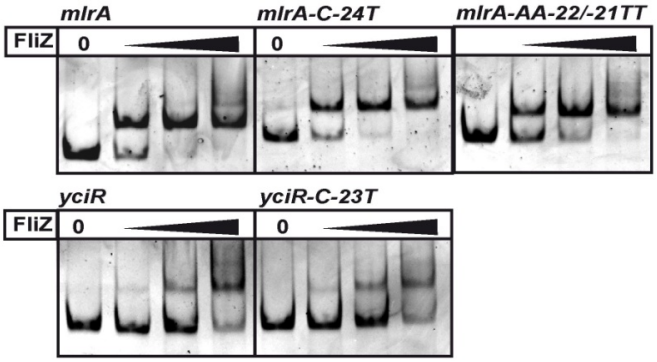

Fig. 4.22: The CAAAACTG motif present in the *mlrA* and *yciR* promoters is not essential for FliZ binding. Mutations were introduced at several sites of the CAAAACTG sequence motif within the *mlrA* and *yciR* promoters and DNA-fragments containing wild-type and mutated promoters were incubated with purified FliZ protein (20, 40, 80 nM) in electrophoretic mobility shift assays.

4.2.2 FliZ acts by sigma factor mimicry

4.2.2.1 The sequence element most important for σ^S selectivity of a promoter also plays a role in FliZ binding

How is FliZ able to distinguish between the very similar σ^{70}- and σ^S-dependent promoters? The analogous question, i.e. how the housekeeping sigma factor σ^{70} and the stress sigma factor σ^S themselves differentially recognize the promoters of their respective regulons has puzzled scientists for years.

As described in detail in section 1.1.3.3 of the introduction, certain promoter sequence elements as well as co-regulating transcriptional regulators are now known to determine σ^S selectivity. Among these, the most important promoter element contributing to σ^S selectivity is a cytosine at position -13 with respect to the transcriptional start site, while σ^{70}-dependent promoters show a bias for guanine at this position (Becker and Hengge-Aronis 2001; Gaal et al. 2001; Weber et al. 2005). A thymine at the neighbouring position -14 also contributes to σ^S selectivity, although to a lesser extent (Becker and Hengge-Aronis 2001). Since C(-13) and T(-14) are both present in the *mlrA* promoter (Fig. 4.23A), mutations were introduced to test their contribution to FliZ binding. Mutation of this TC(-14/-13) element strongly reduced FliZ binding to the *mlrA* promoter (Fig. 4.23B), indicating that this sequence element also plays an important role in FliZ binding to the *mlrA* promoter. The thymines at position -12 and -7, which confine the core -10 hexamer, also contribute to FliZ binding, since *mlrA* promoter fragments carrying mutations at these positions bound FliZ less efficiently (Fig. 4.23B). In contrast, mutating the thymine at position -6 did not affect FliZ binding (Fig. 4.23B). In

conclusion of these experiments and the ones shown in Fig. 4.22, the specific FliZ binding site could be narrowed down to the extended -10 region, comprising the -10 hexamer and the adjacent TC(-14/-13). Thus, FliZ specifically recognizes a promoter element that strongly contributes to σ^S selectivity of a promoter.

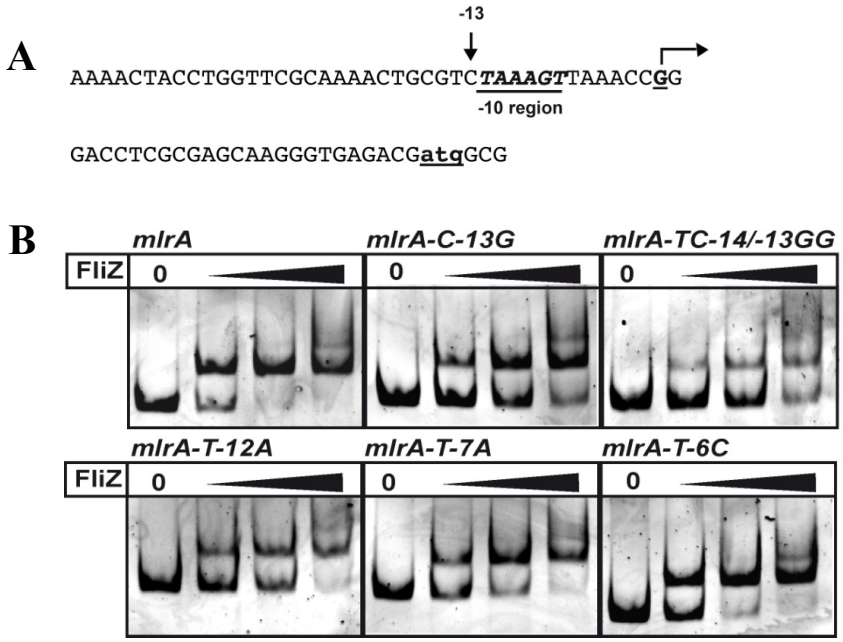

Fig. 4.23: *mlrA* promoter features involved in FliZ binding. Several sites in the *mlrA* promoter sequence **(A)** were mutated and their relevance for FliZ binding was tested in electrophoretic mobility shift assays using DNA-fragments containing wild-type and mutated promoters and purified FliZ protein (20, 40, 80 nM) **(B)**.

4.2.2.2 FliZ mimics σ^S in its promoter recognition mechanism

The results presented above show that FliZ is able to specifically recognize a hallmark of σ^S-dependent promoters. The C(-13) (and probably also T(-14)) is directly contacted by a lysine (K173) located in the first α-helix of domain 3 of σ^S that contacts the extended -10 promoter element (Becker and Hengge-Aronis 2001). σ^{70} contains a glutamate residue at the corresponding position (E458), explaining the different preference of σ^S and σ^{70} for the nucleotide at position -13 (see figure 1.3).

In order to elucidate how FliZ contacts the C(-13) within the *mlrA* promoter, the protein was analyzed for potential similarities with σ^S that might account for a similar DNA binding mechanism. While FliZ does not show overall similarity with σ^S, closer analysis identified a predicted α-helix within FliZ that shows striking similarity to the α-helix in domain 3 of σ^S, which contacts the -10 element (Fig. 4.24A). In particular, FliZ also features a positively charged amino acid (R108) at the position corresponding to K173 in σ^S and the adjacent

amino acids are identical to the ones in σ^S. In σ^S, a number of positively charged amino acids are positioned on one face of the α-helix that contacts the DNA and FliZ carries positively charged residues at several corresponding positions. Due to this striking similarity to the extended -10 recognition element in σ^S, the role of FliZ-R108 in *mlrA* promoter binding was analyzed. Interestingly, a FliZ mutant protein in which the arginine (R108) was replaced by an alanine was strongly reduced in its ability to bind to the *mlrA* promoter (Fig. 4.24B). This was specifically due to the R108A mutation and not to structural instability of the FliZ-R108A mutant, since both FliZ variants showed highly similar digestion patterns and kinetics in limited proteolysis experiments, indicating that both proteins exhibit similar overall stabilities (Fig. 4.24C).

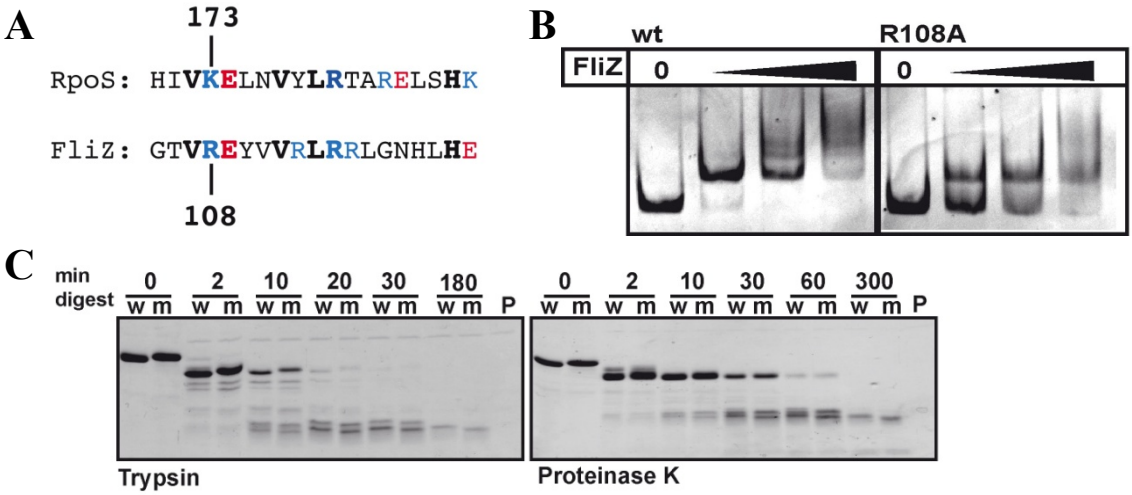

Fig. 4.24: FliZ and σ^S use similar elements for binding to σ^S-dependent promoters. (A) Alignment of a putative alpha helix in the C-terminal region of FliZ with the first alpha helix in domain 3 of σ^S (RpoS). Positively charged residues are printed in blue, negatively charged residues in red. Positions with identical amino acids and positions that are positively or negatively charged in both proteins are indicated by bold letters. **(B).** FliZ (wt) and FliZ-R108A binding to DNA-fragments containing *mlrA* promoter DNA was compared in electrophoretic mobility shift experiments (80, 160, 320 nM FliZ). **(C)** Limited proteolysis of purified FliZ (w) and FliZ-R108A (m). Purified proteins used in the electrophoretic mobility shift assays shown in (B) were incubated with either trypsin or proteinase K for increasing time intervals and digestion products were analyzed by SDS-PAGE with subsequent coomassie staining.

In order to test the effect of the R108A mutation *in vivo*, expression of a *yciR*::*lacZ* fusion was compared in a strain carrying a vector control to expression in strains expressing either wild-type FliZ or the FliZ-R108A mutant from a low copy plasmid. While wild-type FliZ strongly repressed *yciR* expression, *yciR* expression was similar in the strain carrying the vector control and the strain expressing FliZ-R108A (Fig. 4.25A). Western blot analysis demonstrated that both FliZ variants were expressed at similar levels (Fig. 4.25B), thus indicating that FliZ-R108A is unable to repress *yciR in vivo*.

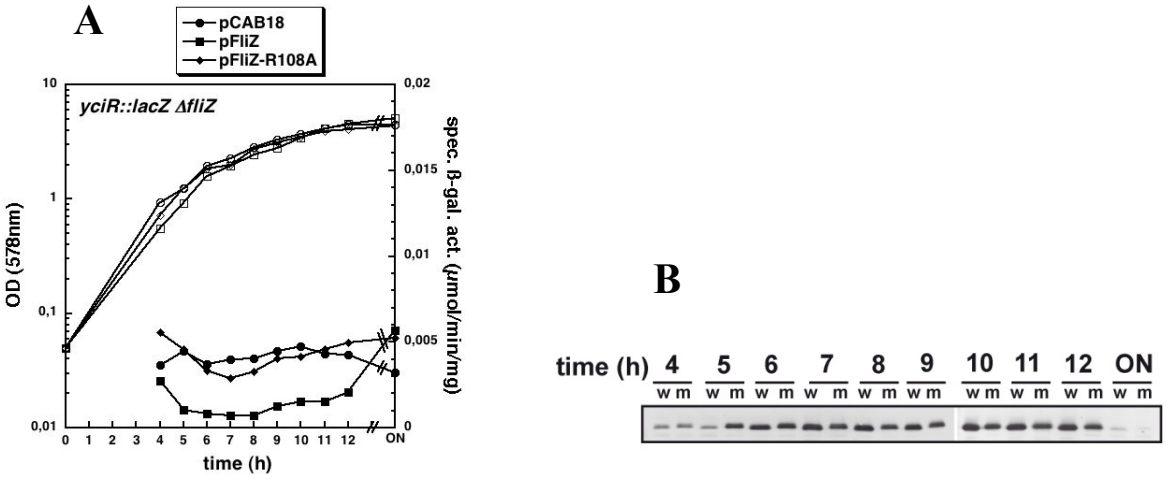

Fig. 4.25: The R108A mutation in FliZ eliminates its repression of the σ^S-dependent gene *yciR*. (A) Expression of a single copy translational *lacZ* fusion to the *yciR* gene in W3110 Δ*fliZ* mutant derivatives carrying the vector control (pCAB18) or expressing wild-type FliZ or the FliZ-R108A mutant from the low copy plasmid. Specific ß-galactosidase activities were determined along the growth curve and in overnight cultures (ON) in cells grown in LB medium supplemented with ampicillin at 28°C. In order to reach similar expression levels of FliZ and FliZ-R108A protein, expression of FliZ-R108A was induced by addition of 10µM IPTG at an OD_{578nm} of 0.5. **(B)** In parallel, cellular levels of FliZ (w) and FliZ-R108A (m) were determined by immunoblot analysis using a polyclonal antibody against FliZ. Figure (B) is assembled from two separate blots.

Taken together the *in vivo* and *in vitro* data presented in this part strongly indicate that FliZ antagonizes σ^S by binding directly to the extended -10 promoter region of σ^S-dependent promoters, and for this interaction employs a structural element (containing R108) that mimics the extended -10 element recognition helix in σ^S (containing key residue K173).

4.2.3 The regulatory mechanism of FliZ might not be restricted to DNA binding

The data presented above clearly demonstrated that FliZ interferes with σ^S-dependent gene expression by binding to σ^S-dependent promoters. However, results obtained in the course of the above studies suggested, that the regulatory potential of FliZ might not be restricted to its DNA-binding activity:

When the bacterial two-hybrid system was used to examine a putative interaction between FliZ and σ^S, σ^{70} was also used as a bait protein to test for a potential interaction with FliZ. Surprisingly, reporter cells co-transformed with the bait plasmid expressing σ^{70} and the target plasmid expressing FliZ were able to grow on selective medium containing the His3 inhibitor 3-AT (Fig. 4.26), indicating an interaction between FliZ and σ^{70}. Growth on selective

medium was detected at both 28°C and 37°C but was slow compared to growth of cells co-expressing σ^S and RssB, which were used as a strongly interacting positive control. Reporter cells co-transformed with the bait plasmid expressing σ^{70} and the vector control pTRG did not grow on selective medium. Thus, these data indicate that FliZ may interact with σ^{70} *in vivo*, but that this interaction is relatively weak. Several *in vitro* protein-protein interaction assays, including different co-immunoprecipitation approaches, native gel electrophoresis and electrophoretic mobility shift assays with both proteins were used in an attempt to confirm these results. However, due to incompatible requirements ensuring stability of the two proteins and vast differences in their isoelectric points (FliZ: 9.2; RpoD: 4.69), all approaches to detect an interaction between FliZ and σ^{70} *in vitro* failed based on methodical problems. Nevertheless, this potential interaction between FliZ and σ^{70} suggests (i) that in addition to binding to DNA, FliZ might work through protein-protein interaction to affect other regulatory processes and (ii) that FliZ might play a role in regulating sigma factor activities that goes beyond inhibition of σ^S. Thus, it will be worth trying to further optimize the *in vitro* approaches to analyse these possibilities.

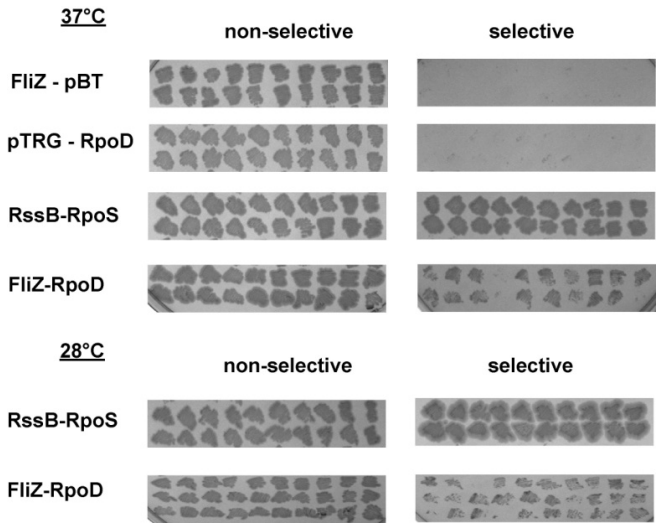

Fig. 4.26: FliZ weakly interacts with σ^{70} *in vivo*. Growth of reporter cells co-transformed with a vector control and/or derivatives of pBT and pTRG plasmids expressing fusions of the indicated proteins to the λ repressor (σ^S (RpoS), σ^{70}(RpoD)) or RNAP αNTD (FliZ, RssB), respectively, in the absence (non-selective) and presence (selective) of the His3 inhibitor 3-AT at 37°C and 28°C as indicated. For further information on experimental details see the Material and Methods section.

4.2.4 FliZ influences motility through at least two different pathways

4.2.4.1 FliZ is a negative regulator of motility in *E. coli*

In addition to the effects on σ^S-dependent gene expression described here, several groups have observed FliZ effects on motility in *E. coli* (Mytelka and Chamberlin 1996; Girgis et al. 2007) and *Salmonella* (Ikebe et al. 1999; Kutsukake et al. 1999; Saini et al. 2008). The reports varied with respect to both the nature of the effects observed and the suggested mechanisms by which FliZ affects motility and/or flagellar gene expression.

To gain a better understanding of the role of FliZ in motility regulation in *E. coli*, swimming motility of W3110 wild-type cells, a non-polar Δ*fliZ* mutant and a strain expressing FliZ from a low copy plasmid was compared. The Δ*fliZ* mutant showed a subtle but reproducible increase in motility as compared to the wild-type, while FliZ expression from the low copy plasmid reduced motility (Fig. 4.27), indicating that in *E. coli*, FliZ acts as a weak negative regulator of motility.

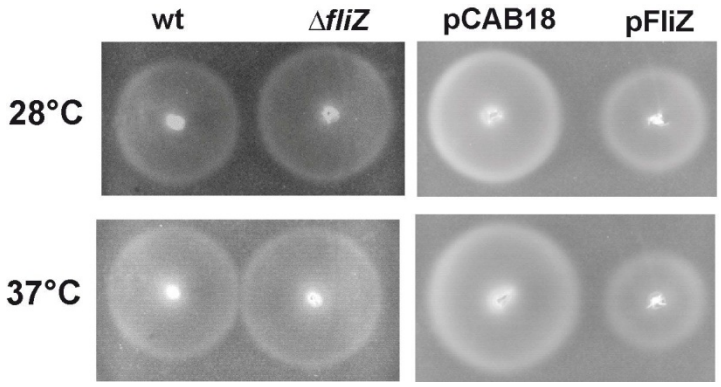

Fig. 4.27: FliZ negatively regulates motility in *E. coli*. Motility of W3110 wild-type (wt) cells, a Δ*fliZ* mutant and strains carrying either the vector control (pCAB18) or expressing FliZ from a low copy plasmid (pFliZ) was tested on motility plates incubated at 28°C and 37°C, as indicated, for 3-5 hours.

As described above, flagellar assembly is based on the hierarchical expression of three classes of flagellar genes. In addition, flagellar gene expression and flagellar activity are subject to many other regulatory influences, the latter, for example, is controlled by c-di-GMP. In order to analyse on which level FliZ interferes with motility, potential effects on flagellar gene expression were tested by measuring the activity of single copy chromosomal *lacZ*-fusions to representatives of all three flagellar gene classes in wild-type and Δ*fliZ* mutant cells. Expression of a fusion to the promoter of the class I *flhDC* operon was elevated in the Δ*fliZ* mutant as compared to the wild-type (Fig. 4.28A). Higher expression in the Δ*fliZ* background

was also observed for fusions to the class II *flgAMN* promoter and to the class III gene *yhjH* (Fig. 4.28B/C), corroborating that FliZ interferes with motility by repressing flagellar gene expression. Thus, FliZ establishes negative feedback control of motility, which also results in elevated class II *fliZ::lacZ* expression in the *fliZ* mutant, i.e. in negative autoregulation (Fig. 4.28D).

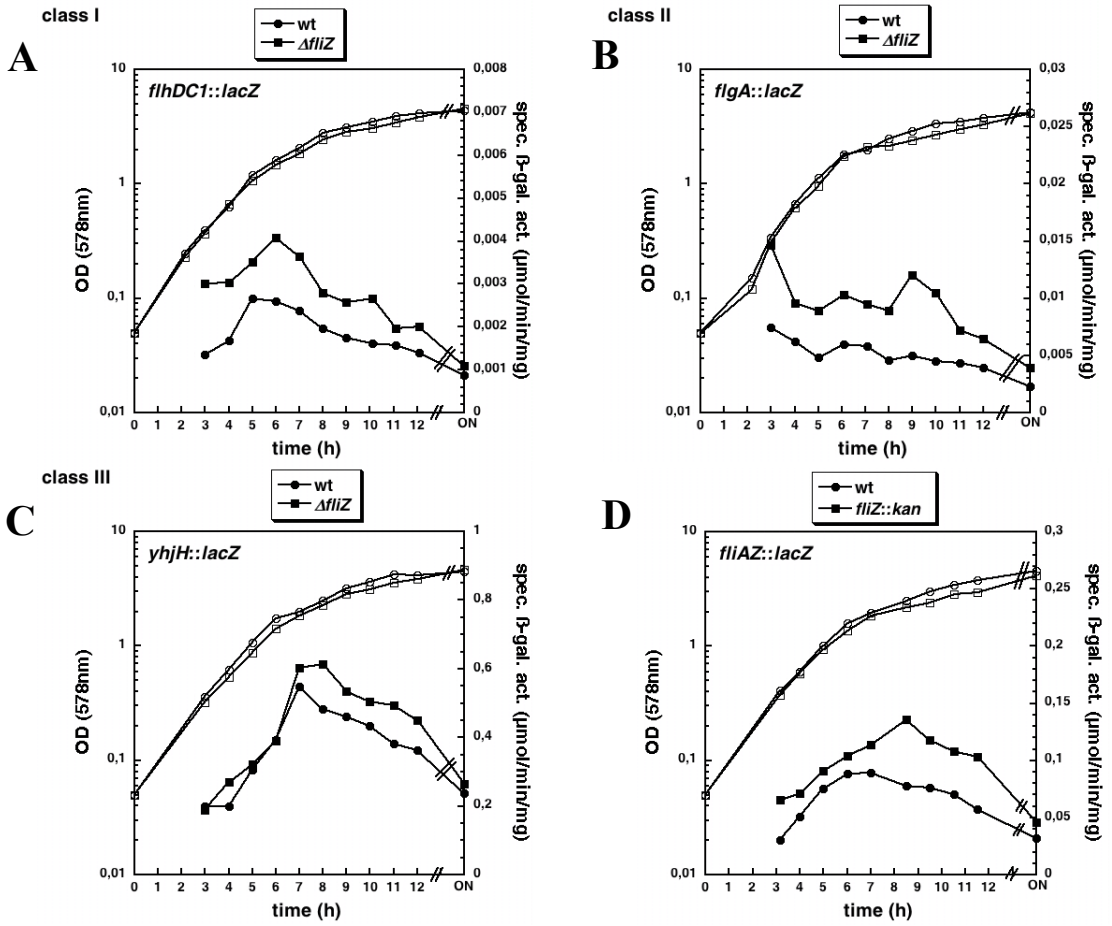

Fig. 4.28: FliZ negatively regulates the expression of flagellar genes. W3110 wild-type (wt) and Δ*fliZ* or *fliZ::kan* mutant derivatives, carrying transcriptional (A, B, D) or translational (C) single copy chromosomal *lacZ* fusions to the indicated flagellar genes, were grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON). The *flhDC1::lacZ* fusion contains the sequence of the entire upstream regulatory region of the *flhDC* operon including the IS5 element.

4.2.4.2 FliZ directly represses flagellar gene expression by binding to a σ^S-promoter-like element within the upstream regulatory region of the *flhDC* operon

As described above, σ^S participates in the down-regulation of flagellar gene expression (see 4.1.3). Thus it seemed possible that the observed FliZ-dependent effects on the expression of

flagellar genes are due to FliZ interfering with σ^S activity. Alternatively, FliZ might influence flagellar gene expression through another mechanism that acts independently of σ^S.

In order to test if FliZ directly interferes with *flhDC* expression, binding of FliZ to the *flhDC* promoter region was tested by EMSA. FliZ bound to a DNA fragment containing the promoter region of the *flhDC* operon (*flhDC*-long, Fig. 4.29A).

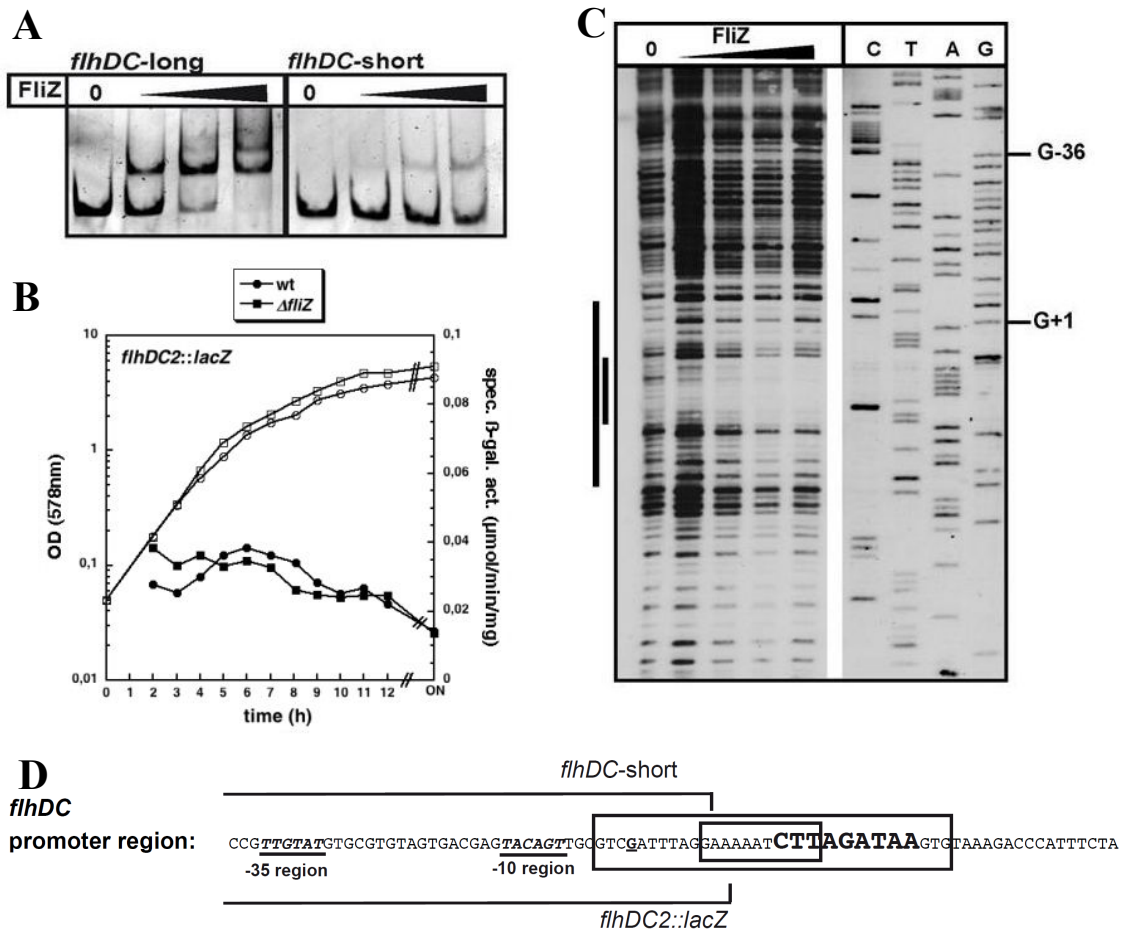

Fig. 4.29: FliZ represses *flhDC* expression by binding to a σ^S-promoter-like element downstream of the *flhDC* promoter. **(A)** Binding of FliZ to DNA fragments with (*flhDC*-long) or without (*flhDC*-short) the σ^S-promoter-like element downstream of the *flhDC* transcriptional start site was compared by EMSA. **(B)** Expression of a single copy transcriptional *lacZ* fusion to the *flhDC* promoter that does not contain the σ^S-promoter-like element downstream of the *flhDC* transcription initiation site (*flhDC2::lacZ*) in W3110 wild-type (wt) and Δ*fliZ* mutant cells grown in LB medium at 28°C. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in the overnight cultures (ON). Note that expression of the *flhDC2::lacZ* fusion is generally higher than the expression of a *lacZ* fusion to the entire *flhDC* upstream regulatory region (*flhDC1::lacZ* in Fig. 4.28 and Fig. 4.30), which is most likely due to the absence of the binding sites of the repressors OmpR and RcsAB in the *flhDC2::lacZ* fusion. **(C)** The FliZ-binding site in the *flhDC* upstream regulatory region was determined by non-radioactive DNaseI footprint analysis and the binding site was mapped to the promoter sequence **(D)**. A core binding site and potential upstream and downstream extensions are indicated by smaller and larger bars (C) and boxes (D), respectively. -10 and -35 regions are indicated by bold italic letters, the transcriptional start site (Soutourina et al. 1999; Wei et al. 2001; Barker et al. 2004) is printed as a bold, underlined letter and the σ^S-promoter-like element downstream of the *flhDC* promoter is printed in larger, bold letters. Numbers in (C) indicate positions relative to the transcriptional start site. The sequencing reactions were performed with the same labelled primer used for the footprint experiments. The end point of the region present in the *flhDC2::lacZ* fusion used in (B) and the end point of the *flhDC*-short DNA fragment used in (A) are also indicated in (D).

This was very surprising since the *flhDC* promoter is known to be σ^{70}-dependent and the above results had shown that FliZ specifically binds to σ^{S}-dependent promoters. However, closer analysis of the *flhDC* promoter region revealed the presence of a short sequence just downstream of the transcriptional start site that resembles the extended -10 element of a σ^{S}-dependent promoter (Fig. 4.29D). To test whether FliZ binds to this "σ^{S}-promoter-like element", a shorter DNA fragment, which contained the σ^{70}-dependent *flhDC* promoter but lacked this element (Fig. 4.29D), was tested in EMSA. Interestingly, FliZ did not bind to this fragment (Fig. 4.29A) and expression of a transcriptional *lacZ* fusion to the *flhDC* promoter, which did not contain this element (*flhDC2::lacZ*, Fig. 4.29D), did not show regulation by FliZ (Fig. 4.29B), thus revealing the importance of the -10 element-like sequence for FliZ binding. Moreover, DNaseI footprint experiments with the *flhDC* promoter region confirmed that FliZ binds downstream of the *flhDC* promoter overlapping with the σ^{S}-promoter-like element (Fig. 4.29C/D). Together these results demonstrate that FliZ interferes with *flhDC* expression by binding to a sequence downstream of the transcriptional start site, which resembles the extended -10 element of a σ^{S}-dependent promoter.

It seemed possible that the putative -10 element is part of a σ^{S}-dependent promoter that is used to drive expression of flagellar genes under certain yet unknown conditions, e.g. in a biofilm. To test this, a transcriptional *lacZ* reporter fusion to a region containing the σ^{S}-promoter-like element as well as 40 nucleotides upstream, but missing the entire *flhDC* promoter was constructed (*flhDC3::lacZ*) (Fig. 4.30A). However, no ß-galactosidase activity could be detected in cells carrying this fusion, neither after growth on plates for several days (Fig. 4.30B), nor during growth in liquid culture (Fig. 4.30C). Thus, this element does not seem to be part of a σ^{S}-dependent promoter. These results indicate that for regulating *flhDC* expression, FliZ uses a sequence with similarity to the extended -10 region of σ^{S}-dependent promoters as a regular operator site, thereby extending its regulatory influence to a gene activated by a sigma factor different from σ^{S}.

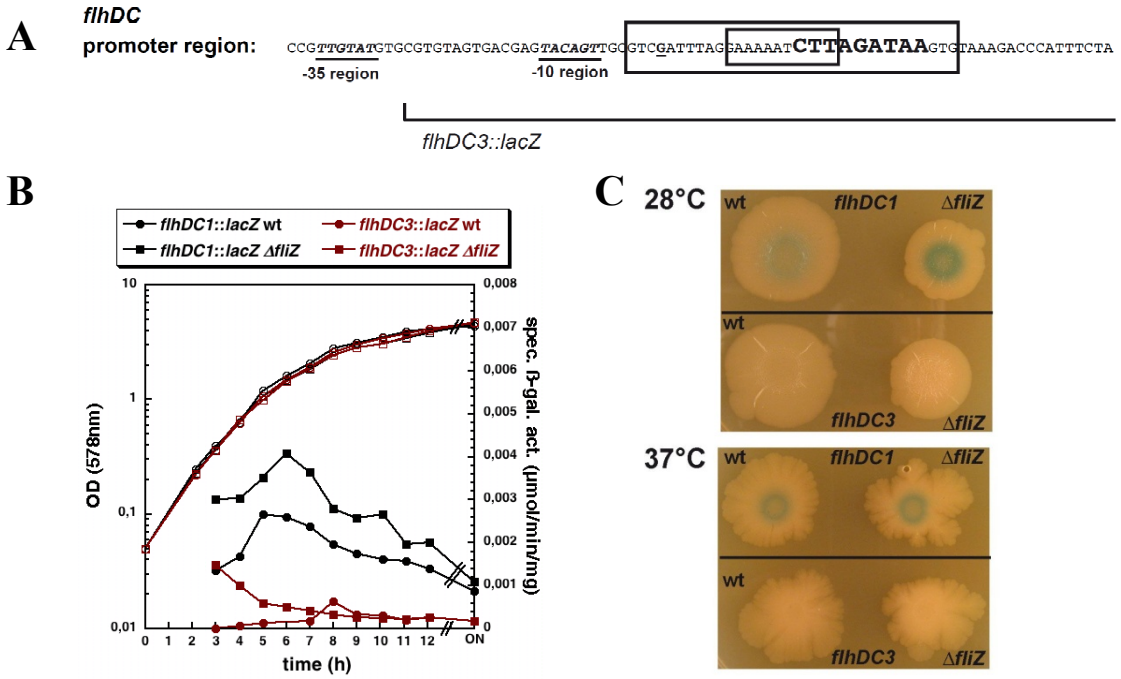

Fig. 4.30: The σ^S-promoter-like element in the *flhDC* upstream regulatory region does not constitute a σ^S-dependent promoter. (A) The start point of the *flhDC3::lacZ* fusion used in (B) is indicated in the promoter sequence of the *flhDC* operon. -10 and -35 regions, the transcriptional start site, the FliZ binding site and the σ^S-promoter-like element are indicated as in Fig. 4.29D. **(B & C)** The expression of this *lacZ* fusion was compared to expression of a transcriptional *lacZ* fusion to the entire *flhDC* promoter region (*flhDC1::lacZ*) in W3110 wild-type (wt) and Δ*fliZ* mutant cells grown in LB medium at 28°C (B) or on LB/agar plates containing X-gal (C) at 28°C and 37°C as indicated. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) for cultures in (B) were determined along the growth curve and in overnight cultures (ON). Cells in (C) were grown for 7 days before photographs were taken. Note that the specific β-galactosidase activity measured for the *flhDC3::lacZ* fusion in (B) reflects background levels detected in these assays.

Since FliZ also affected the expression of class II and III genes (Fig. 4.28B-D), binding of FliZ to promoter regions of genes belonging to these classes was tested by ELISA. However, FliZ did not bind to the class II and class III promoters tested (Fig. 4.30), suggesting that the FliZ effect on expression of the master regulator operon *flhDC* is relayed to the class II and III genes.

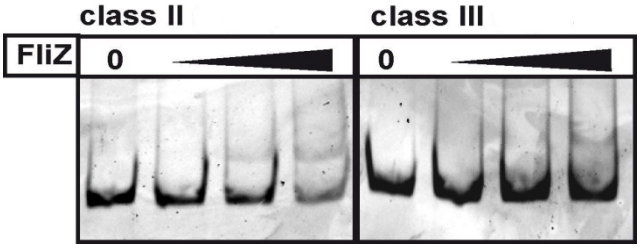

Fig. 4.31: FliZ does not bind to the promoter regions of flagellar class II and III genes. Binding of FliZ (20, 40, 80 nM) to DNA fragments containing the class II promoter of the *flgA* gene and the class III promoter of the *flgM* gene was tested by EMSA.

4.2.4.3 FliZ indirectly influences motility through regulation of σ^S-dependent phosphodiesterases

While this work was in progress, the *fliZ* gene was identified in a screen for suppressor mutations that restore the motility defect of a *yhjH* mutant (Girgis et al. 2007). Figure 4.32 shows, that a *ΔfliZ* mutation also had this effect in strain W3110 under the conditions used here to monitor motility. Interestingly, this effect could only be observed at 28°C, but not at 37°C. Thus, one of the regulators contributing to this phenotype seems to be subject to temperature regulation.

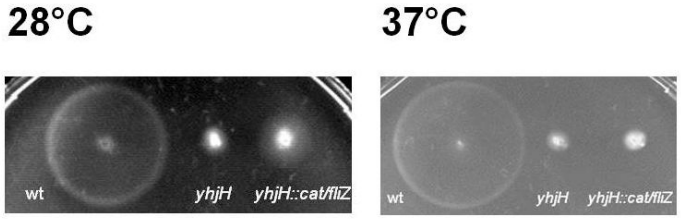

Fig. 4.32: A *fliZ* mutation suppresses the non-motile phenotype of a *yhjH* mutant at 28°C, but not at 37°C. Motility of W3110 wild-type (wt) cells, Δ*yhjH* and Δ*yhjH*/Δ*fliZ* mutant derivatives was tested on motility plates incubated at 28°C and 37°C, as indicated, for 4-6 hours.

As described above, *yhjH* codes for a phosphodiesterase that is essential for motility in *E. coli*, as it degrades cyclic di-GMP that otherwise would interfere with motility. One possible scenario explaining suppression of the motility defect of the *yhjH* mutant by mutation of *fliZ* was that a mutation in *fliZ* results in higher expression of one or several σ^S-dependent phosphodiesterases during post-exponential phase. This may compensate for the loss of YhjH phosphodiesterase activity and thereby partially restore motility in the *yhjH/fliZ* double mutant. Expression of the *yciR* gene, encoding a σ^S-dependent phosphodiesterase involved in regulation of curli fimbriae expression, was shown above to be repressed in a strain expressing *fliZ* from a low copy plasmid (Fig. 4.3C). Consistently, a *yciR::lacZ* fusion showed higher post-exponential expression in a Δ*fliZ* mutant (Fig. 4.33A). In addition, previous studies and studies performed in the Hengge group in parallel with this work had revealed that in *E. coli*, the majority of GGDEF and EAL-genes are under the control of σ^S (Weber et al. 2006; Sommerfeldt et al. 2009), thus making them potential targets for regulation by FliZ. Therefore, expression of five additional σ^S-dependent genes encoding proteins with an EAL domain was monitored in a Δ*fliZ* mutant (note, however, that expression of the *rtn* gene is negatively controlled by σ^S (Sommerfeldt et al. 2009)) (Fig. 4.33A).

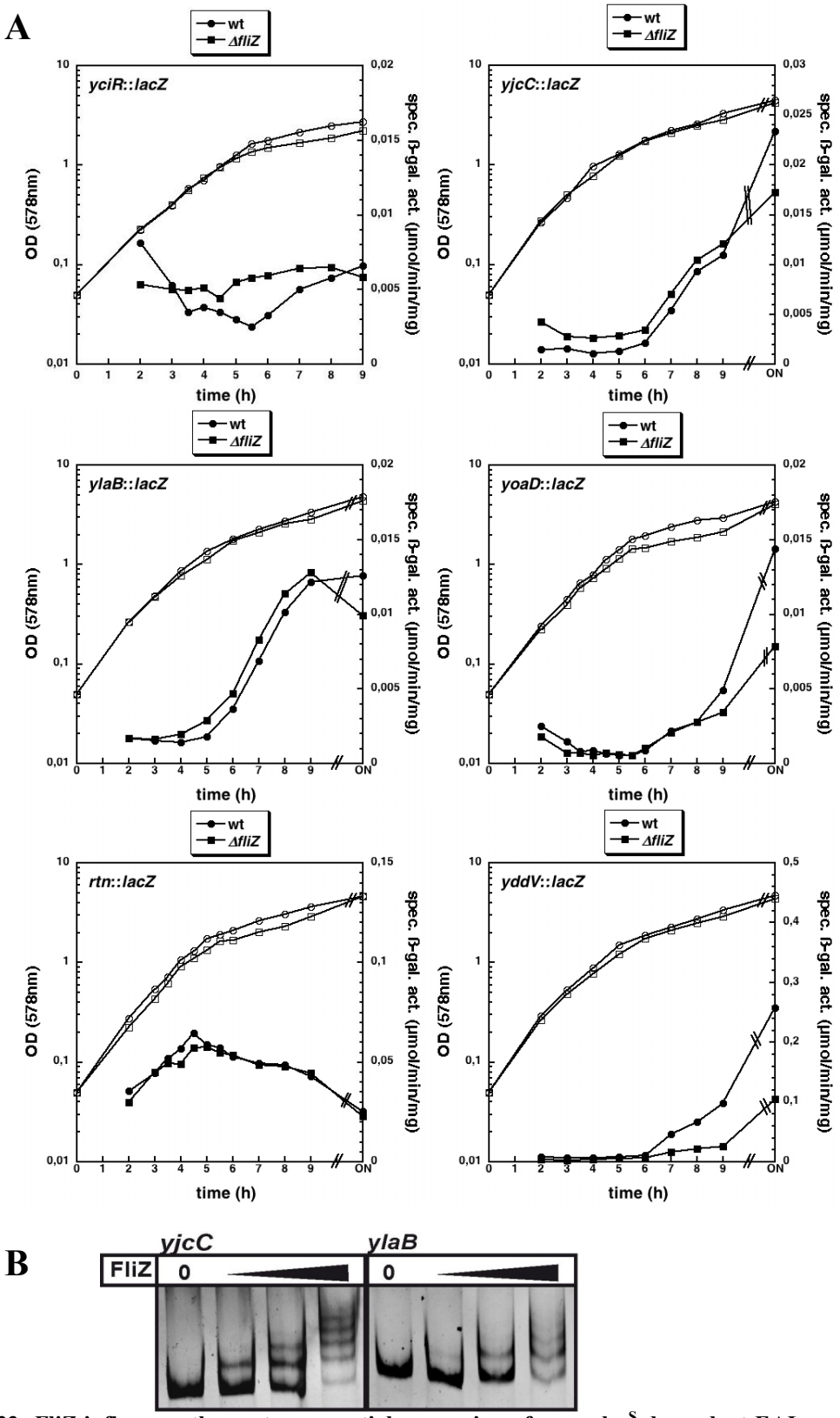

Fig. 4.33: FliZ influences the post-exponential expression of several σ^S-dependent EAL-genes. (A) W3110 wild-type (wt) and Δ*fliZ* mutant derivatives carrying single copy chromosomal *lacZ* fusions to the indicated σ^S-dependent EAL genes were grown in LB medium at 28°C. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined during exponential, post-exponential, early stationary growth and in overnight cultures (ON). Note that the PDE YddU is encoded in an operon together with the DGC YddV. Since *yddV* is the first gene in the *yddVU* operon, a *yddV::lacZ* fusion was used to monitor expression of this operon. **(B)** Binding of FliZ (20, 40, 80 nM) to DNA fragments carrying promoter regions of *yjcC* and *ylaB* was tested by EMSA.

Expression of *lacZ* fusions to *yjcC* and *ylaB* was induced slightly earlier in post-exponential phase in the Δ*fliZ* mutant, while expression of *lacZ* fusions to *yoaD* and to the *yddV* gene, which is the first gene in an operon together with the EAL gene *yddU*, was reduced in late post-exponential and stationary phase in the Δ*fliZ* mutant background. In contrast, expression of a *lacZ* fusion to the negatively σ^S-dependent EAL-gene *rnt* remained unchanged in the Δ*fliZ* mutant as compared to the wild-type. Since the *yjcC* and *ylaB* genes are activated by σ^S and negatively regulated by FliZ, they display the typical regulation of many direct FliZ targets. Thus, binding of FliZ to promoter DNA of these genes was tested by EMSA. Although FliZ seemed to weakly bind to both promoters, binding was not as efficient as e.g. to the *mlrA* or *gadE* promoters (compare to Fig. 4.17 and Fig. 4.18) and it therefore remained unclear whether FliZ-mediated repression of these genes is direct or indirect.

Together these results show, that FliZ directly (at least in the case of *yciR*) and indirectly influences the expression of several σ^S-dependent EAL-genes. If these genes encode functional phosphodiesterases that are active under the conditions tested, altered expression in the *fliZ* mutant can be expected to result in changes in the c-di-GMP pools influenced by these enzymes and thus in effects on c-di-GMP-dependent phenotypes, possibly including motility.

In order to test the hypothesis that suppression of the motility defect of the *yhjH* mutant by mutation of *fliZ* is based on the observed alterations in expression levels of σ^S-dependent EAL genes, triple mutants defective in *yhjH*, *fliZ* and either one of the six σ^S-dependent EAL-genes were constructed. While introduction of the *fliZ* mutation into the *yhjH* mutant partially restored motility at 28°C, additional introduction of a *yciR* mutation reversed this effect and completely abolished motility, indicating that *yciR* is responsible for FliZ-mediated restoration of motility (Fig. 4.34). Introduction of a *yjcC* mutation into the *yhjH/fliZ* mutant background had a similar effect, and introduction of a *ylaB* mutation slightly reduced motility of the *yhjH/fliZ* double mutant, demonstrating that *yjcC* and (to a lesser extend) *ylaB* also participate in restoring motility of the *yhjH* mutant in the absence of FliZ (Fig. 4.34).

Interestingly, the three EAL-genes found to be involved in FliZ-mediated suppression of the motility defect of the *yhjH* mutant were the ones that showed (slightly) higher post-exponential expression in the Δ*fliZ* mutant (Fig. 4.33). Single mutations in none of the tested σ^S-dependent EAL-genes affect motility (Nicole Sommerfeldt-Impe and Regine Hengge, unpublished results). Thus, earlier induction of *yciR*, *yjcC* and *ylaB* in the *fliZ* mutant background seems to alter the functional context of the encoded EAL proteins, since they are

able to affect motility in the *fliZ* mutant but not in an otherwise wild-type background. In this context it should be noted that double mutants defective in *yhjH* and either *yciR*, *yjcC* or *ylaB* appeared to be even less motile than the *yhjH* mutant, which displayed some faint motility after the increased incubation time used in this assay (Fig. 4.34). This demonstrates that these three EAL genes are generally capable of affecting motility. However, their influence on motility significantly increases with elevated post-exponential expression in the *fliZ* mutant.

Surprisingly, the *yhjH/fliZ/rtn* triple mutant was even more motile than the *yhjH/fliZ* double mutant, but a mutation in *rtn* did not suppress the motility defect of the *yhjH* mutant when introduced in the presence of *fliZ* (i.e. in the *yhjH/rtn* double mutant). This suggests that the Rtn-mediated effect requires altered expression of another FliZ-dependent gene, (e.g. a phosphodiesterase that is repressed by Rtn), which is consistent with the unaltered expression level of the *rtn* gene itself in the Δ*fliZ* mutant (Fig. 4.33A).

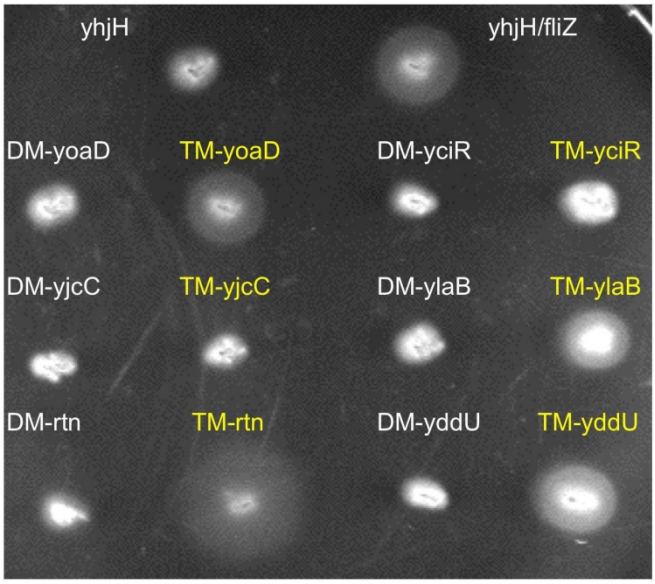

Fig. 4.34: FliZ-regulated EAL-genes contribute to FliZ-mediated suppression of the motility defect of the *yhjH* mutant. Motility of a W3110 Δ*yhjH* single mutant, a Δ*yhjH*/Δ*fliZ* double mutant and double (DM) and triple mutants (TM) carrying mutations in *yhjH* or *yhjH/fliZ*, respectively, together with mutations in either one of the indicated σ^S-dependent EAL genes. Motility was tested on motility plates incubated at 28°C for approximately 8 hours.

Higher expression of σ^S-dependent phosphodiesterases in the *fliZ* mutant finally also provided a possible explanation for an as yet unexplained observation made with this mutant: As shown in figure 4.35, expression of a *csgB::lacZ* fusion was reduced in the Δ*fliZ* mutant, which was puzzling in view of the above finding that expression of the essential curli activators YdaM and MlrA started earlier and reached higher levels in the *fliZ* mutant (see Fig. 4.5). However, elevated expression of the negative curli regulator YciR in the *fliZ* mutant will likely lead to a

reduction of curli fimbriae expression in this background. Moreover, expression of YhjH, which also has a negative influence on curli fimbriae expression (Gisela Becker in (Pesavento et al. 2008)), is elevated in the *fliZ* mutant (see Fig. 4.28), thus probably contributing to reduction of *csgB::lacZ* expression. In addition to these enzymes, other phosphodiesterases with a potential to influence curli fimbriae expression may show elevated expression in the *fliZ* mutant and thereby contribute to curli fimbriae repression. Since the above results have shown that FliZ-mediated alterations in the expression profiles of these enzymes may change the functional context in which these enzymes work, this might also include phosphodiesterases that do not affect curli fimbriae expression in the wild-type background.

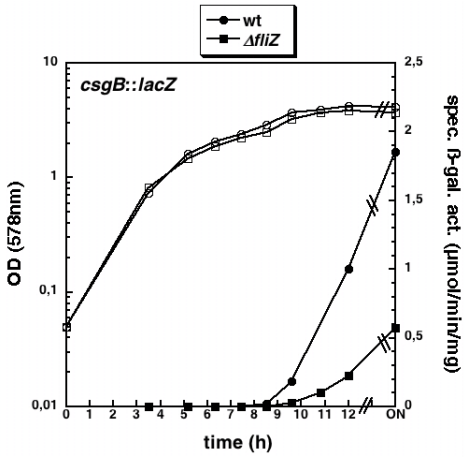

Fig. 4.35: Curli fimbriae expression is reduced in the Δ*fliZ* mutant. Expression of a single copy chromosomal *csgB::lacZ* fusion in W3110 wild-type (wt) and Δ*fliZ* mutant derivatives grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

In summary the data presented in this part demonstrate that FliZ negatively affects motility directly by binding to the *flhDC* promoter, thus repressing flagellar gene expression and indirectly by repressing σ^S-dependent EAL-genes in post-exponential phase, thereby affecting flagellar activity. Since YciR and maybe additional FliZ-regulated phosphodiesterases also influence curli fimbriae expression, this further corroborates the important role of FliZ in the precise timing of gene expression during post-exponential phase and entry into stationary phase. In addition, FliZ binding to a σ^S-promoter-like operator site within the *flhDC* upstream regulatory region shows that FliZ binding is not restricted to promoters activated by σ^S.

4.3 New regulatory aspects in the expression of curli fimbriae and motility

As it can be inferred from the above results, a detailed understanding of the mechanisms controlling curli fimbriae expression and motility is essential for understanding the principles underlying the intricate switch mechanism governing the transition from the planktonic to the adhesive lifestyle. However, several important details on the regulation of both curli fimbriae expression and motility were still missing when this thesis was started. These included e.g. the mechanism through which c-di-GMP-bound YcgR interferes with motility as well as several mechanistic details on the transcriptional regulation of *csgD*. Open questions related to *csgD* transcription concern the identity of the effector through which c-di-GMP synthesized by YdaM activates *csgD* transcription, the location of the MlrA binding site on the *csgD* promoter and the mechanism through which MlrA activates *csgD* transcription.

Moreover, some observations made in the course of the experiments presented above (e.g. the fact that YciR, which was thought to be restricted to controlling CsgD expression is able to influence motility in a *fliZ* mutant background) and data published by other groups (e.g. on the multiple, but partly uncharacterized roles H-NS plays in expression of CsgD (Gerstel et al. 2003; Ogasawara et al. 2010a)) hinted at additional unidentified mechanistic details that may add to a better understanding of c-di-GMP control of curli fimbriae expression and motility. Elucidation of these details may also reveal some general information on the coordination of c-di-GMP signalling in bacterial cells that contain multiple GGDEF and EAL domain proteins. Therefore, some of these aspects were analyzed in more detail and the results will be presented next.

4.3.1 Regulation of curli fimbriae expression and motility by the diguanylate cyclase YdaM and the phosphodiesterase YciR

4.3.1.1 Regulation of curli fimbriae expression by YdaM and YciR involves mechanisms that go beyond simple synthesis and degradation of c-di-GMP

Directly preceding the beginning of this work, results obtained by the Hengge group had shown that the GGDEF protein YdaM and the composite GGDEF-EAL protein YciR antagonistically control *csgD* expression (Weber et al. 2006). The expression of both proteins depends on σ^S and enzymatic assays with the purified proteins had established that YdaM is a

functional diguanylate cylase, while YciR degrades c-di-GMP. Moreover, the wild-type swimming behaviour of *ydaM* and *yciR* mutants, as well as whole genome transcription profiling experiments during entry into stationary phase had indicated that *csgD* transcription is the only target of YdaM/YciR-mediated regulation under these conditions ((Weber et al. 2006) and results by Gisela Becker in (Pesavento et al. 2008)).

The observation that YciR antagonizes YdaM in the control of curli fimbriae expression suggested that it degrades the c-di-GMP synthesized by YdaM, which is essential for *csgD* expression. Yet, in contrast to all other composite GGDEF-EAL proteins in *E. coli* except YegE (which according to genetic data seems to act as a DGC (see above)), YciR possesses a conserved GGDEF motif. This raised the question whether both domains play a role in YciR function. In order to get an overview of the contributions of c-di-GMP synthesizing and degrading activities to the antagonistic control of curli fimbriae expression by YdaM and YciR, mutant versions of YdaM and YciR defective in the active site motifs of the respective GGDEF and EAL domains were tested for their influence on *csgB::lacZ* expression. When YdaM wild-type protein was expressed form plasmid pBAD18 upon induction with 0.05 % arabinose, it restored curli fimbriae expression in a *ydaM::cat* mutant (Fig. 4.36A). Interestingly, residual *csgB::lacZ* expression in the *ydaM* mutant was completely repressed in the control strain carrying the empty vector in the presence of arabinose. Arabinose-suppression of *csgB::lacZ* expression most likely reflects the fact that biofilm formation in *E. coli* is subject to carbon catabolite repression (Jackson et al. 2002), although the molecular targets through which this regulation affects curli fimbriae expression remain to be determined. Over-expression of wild-type YdaM overcomes this arabinose-induced repression. In contrast, *csgB::lacZ* expression was only very weakly induced when YdaM-EE334/335AA, which carries a mutation in the active site of the GGDEF domain that abolishes diguanylate activity, was expressed from the same plasmid (Fig. 4.36A). Thus, YdaM-mediated activation of curli fimbriae expression requires an intact GGEEF motif, corroborating that it affects *csgD* transcription through synthesis of c-di-GMP. However, it is interesting to note that arabinose-induced expression of YdaM-EE334/335AA lead to earlier induction of residual *csgB::lacZ* expression as compared to the strains carrying the empty plasmid or plasmids with wild-type and mutated YdaM in the absence of inducer. Therefore, YdaM might weakly affect curli fimbriae expression through an additional mechanism that does not require its enzymatic activity.

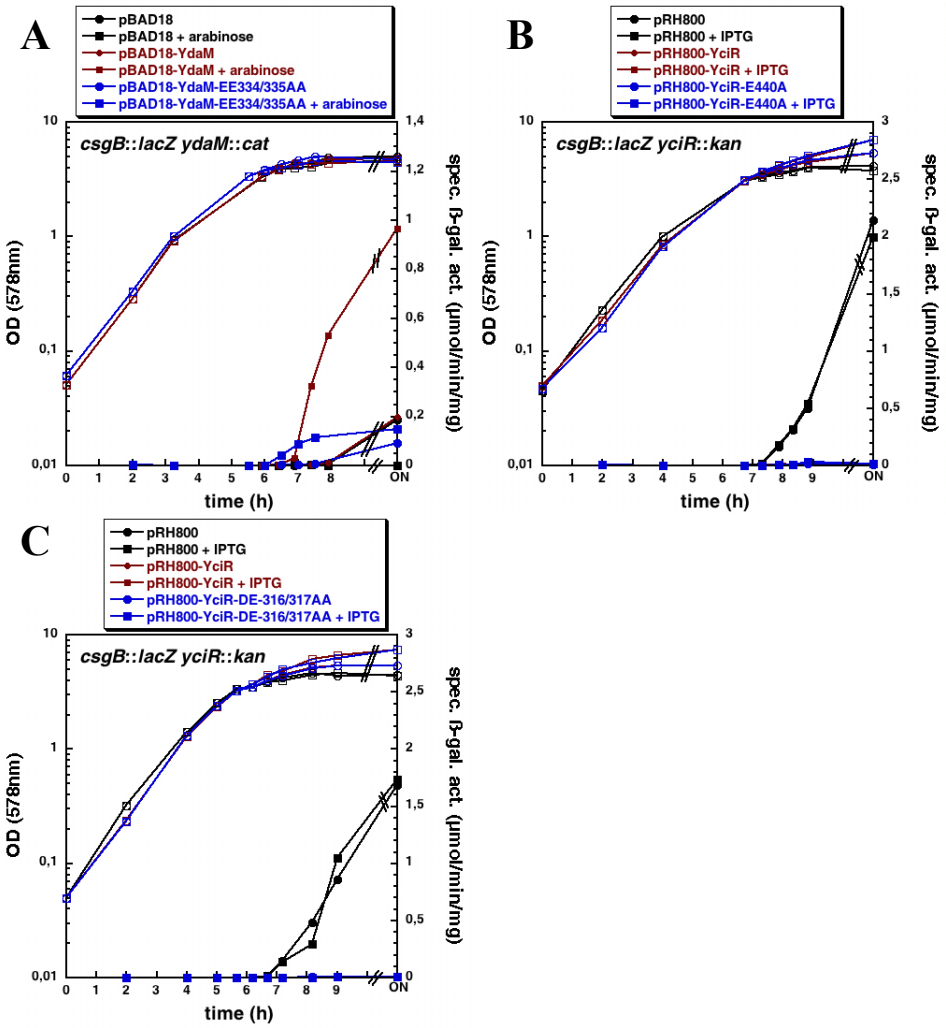

Fig. 4.36: Contributions of the GGDEF and EAL motifs of YdaM and YciR to regulation of curli fimbriae expression. Expression of a single copy chromosomal *csgB::lacZ* fusion in **(A)** a MC4100 *ydaM::cat* mutant carrying either the empty vector pBAD18 or its derivatives expressing wild-type YdaM or the GGDEF-motif mutant YdaM-EE334/335 from the p_{ara} promoter in the presence and absence of the inducer arabinose as indicated, and in **(B &C)** a MC4100 *yciR::kan* mutant carrying either the empty vector pRH800 or its derivatives expressing wild-type YciR, or either the EAL-motif mutant YciR-E440A (B) or the GGDEF-motif mutant YciR-DE-316/317AA (C) from the p_{tac} promoter in the presence or absence of the inducer IPTG as indicated. Cells were grown at 28°C in LB medium supplemented with ampicillin. 0.05 % arabinose (A) or 100 µM IPTG were added at an OD_{578nm} of 3. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

Due to the observed repression of curli fimbriae expression by arabinose, the pBAD vector system could not be used for testing the role of the GGDEF and EAL motifs in mediating the negative effect that YciR exerts on curli fimbriae expression. Instead, wild-type YciR and two YciR variants, in which essential residues of the EAL motif (YciR-E440A) and the GGDEF motif (YciR-DE316/317AA) had been mutated, were expressed from plasmid pRH800 under the control of an IPTG inducible promoter. As expected, expression of wild-type YciR repressed *csgB::lacZ* expression in a *yciR::kan* mutant (Fig. 4.36B). Surprisingly, expression of the YciR mutant carrying a defective EAL active site motif also strongly repressed curli

fimbriae expression, suggesting that the inhibitory effect of YciR on curli fimbriae expression does not require phosphodiesterase activity (Fig. 4.36B). In the context of curli fimbriae expression, YciR therefore seems to act through a different mechanism. While YdaM activates curli fimbriae expression through synthesis of c-di-GMP, the antagonizing effect of YciR does not appear to be based on its ability to degrade the c-di-GMP synthesized by YdaM. Thus, additional features of YciR must enable it to counteract the activity of YdaM. Since mutation of the GGDEF motif did not alleviate repression of *csgB::lacZ* expression by YciR (Fig. 4.36C), a potential DGC activity of this domain does not seem to be essential for this function, either. This does not exclude the possibility that the GGDEF domain plays a role in this function that does not require enzymatic activity, e.g. by mediating a direct interaction between YdaM and YciR. Thus, a detailed analysis of the contributions of both YciR domains to repression of curli fimbriae expression and to a potential interaction between YdaM and YciR is needed to clarify the molecular basis of the antagonistic roles of these two proteins. These analyses are currently conducted in the Hengge group (Sandra Lindenberg, unpublished results).

4.3.1.2 YciR affects curli fimbriae expression and motility through different molecular mechanisms

Since results presented above had shown that elevated post-exponential expression of YciR in the *fliZ* mutant allows it to positively affect motility (see 4.2.4.3), an interesting question arose, as to whether this YciR-mediated effect requires its PDE activity or is instead also based on a PDE-activity-independent mechanism. In order to test the effect of post-exponential expression of the YciR wild-type and GGDEF/EAL mutant proteins on motility, swimming behaviour of strains ectopically expressing these proteins from pRH800 was monitored. Ectopic expression of YciR, but not YciR-E440A, which carries a mutation in the EAL motif, restored motility in the *yhjH* mutant, demonstrating that elevated expression of *yciR* in post-exponentially growing cells compensates for loss of *yhjH* through degradation of cyclic di-GMP (Fig. 4.37). In contrast, mutation of the GGDEF motif did not affect suppression of the motility defect of the *yhjH* mutant by ectopic YciR expression (Fig. 4.37). Together with the above results these data show that YciR affects different c-di-GMP-dependent phenotypes through different molecular mechanisms, establishing the first example of a GGDEF or EAL domain protein that acts through multiple mechanisms, some of which require its enzymatic activity against c-di-GMP, while others work independently of it.

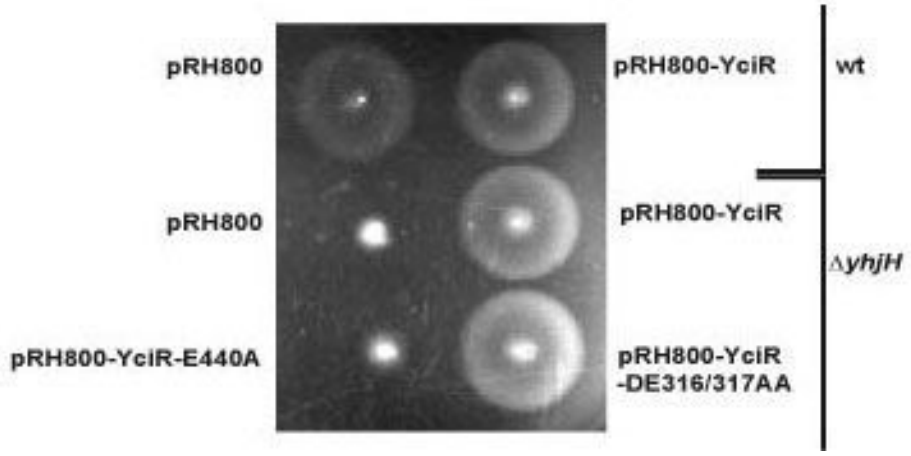

Fig. 4.37: The positive effect of post-exponential YciR expression on motility requires an intact YciR-EAL motif. W3110 wild-type (wt) and Δ*yhjH* mutant strains, carrying either the vector control (pRH800) or expressing wild-type YciR (pRH800-YciR) or YciR mutant proteins defective in the signature motifs of the EAL (pRH800-YciR-E440A) or GDDEF domain (pRH800-YciR-DE316/317AA) from plasmid pRH800, were tested on motility plates supplemented with ampicillin. Plates were incubated at 28°C for approximately 5 hours.

4.3.1.3 Other GGDEF proteins might contribute to activation of curli fimbriae synthesis

The fact that curli fimbriae expression is strongly reduced in the *ydaM* mutant and the possibility that YciR might exert its influence by direct interaction with YdaM suggested a scenario in which YdaM is the major DGC responsible for synthesizing the c-di-GMP required for curli fimbriae expression, while YciR acts by specifically inhibiting YdaM. If the role of YciR in curli fimbriae regulation was restricted to inhibiting c-di-GMP synthesis by YdaM, YciR-dependent curli fimbriae repression should only become apparent in the presence of YdaM. However, expression of a *csgB::lacZ* fusion was reduced in a *yciR/ydaM* double mutant as compared to the *yciR* single mutant, but was still higher than in the wild-type (Fig. 4.38). Therefore, other diguanylate cyclases, which are also subject to inhibition by YciR, may synthesize the c-di-GMP that induces *csgD* transcription in the absence of YciR and YdaM. An alternative explanation is that in addition to its antagonistic effect against YdaM, YciR may exert a basic, c-di-GMP-independent inhibitory effect on MlrA. This may render *csgD* transcription independent of c-di-GMP in the absence of YciR. Both hypotheses are consistent with the observation that YciR still seems to exert a negative influence on curli fimbriae expression in the absence of YdaM, which implies that its role is not restricted to antagonizing YdaM.

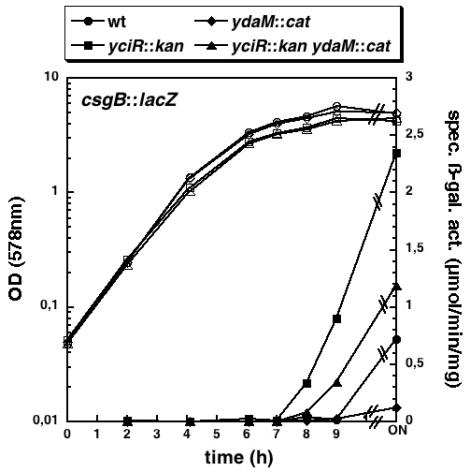

Fig. 4.38: In the absence of YdaM, YciR still exerts a negative influence on curli fimbriae expression. Expression of a single copy chromosomal *csgB::lacZ* fusion in MC4100 wild-type (wt) cells and derivatives carrying the indicated single and double mutations in *ydaM* and *yciR*. Cells were grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

In order to test whether other diguanylate cyclases are able to contribute to induction of curli fimbriae expression in the absence of YciR, expression of a *csgB::lacZ* fusion was compared in single and double mutants defective in *yciR* and selected GGDEF proteins. Like YdaM, the analyzed GGDEF proteins contain GGDEF but no EAL domains and display conserved GGDEF motifs and I-sites, suggesting that they are able to function as DGCs. None of the *yciR*/GGDEF gene double mutants showed a decrease in *csgB::lacZ* expression as compared to the *yciR* single mutant, indicating that none of the corresponding GGDEF proteins seem to contribute to c-di-GMP synthesis relevant for curli fimbriae induction (Fig. 4.39). However, *csgB::lacZ* expression may be maximally induced in the *yciR* mutant and this may not allow for the detection of subtle effects mediated by single GGDEF proteins, particularly if several DGCs collectively contribute to the synthesis of the YdaM-independent c-di-GMP. Note also, that the contribution of the GGDEF-EAL protein YegE, which is required for full induction of curli fimbriae synthesis and down-regulation of motility in strain W3110, has not been analyzed here. Analysis of the role of this protein and the mechanism through which c-di-GMP synthesized by YegE is integrated into YdaM/YciR-mediated regulation of *csgD* transcription was performed by other members of the Hengge group (Gisela Becker in (Pesavento et al. 2008) and Sandra Lindenberg and Regine Hengge, unpublished results).

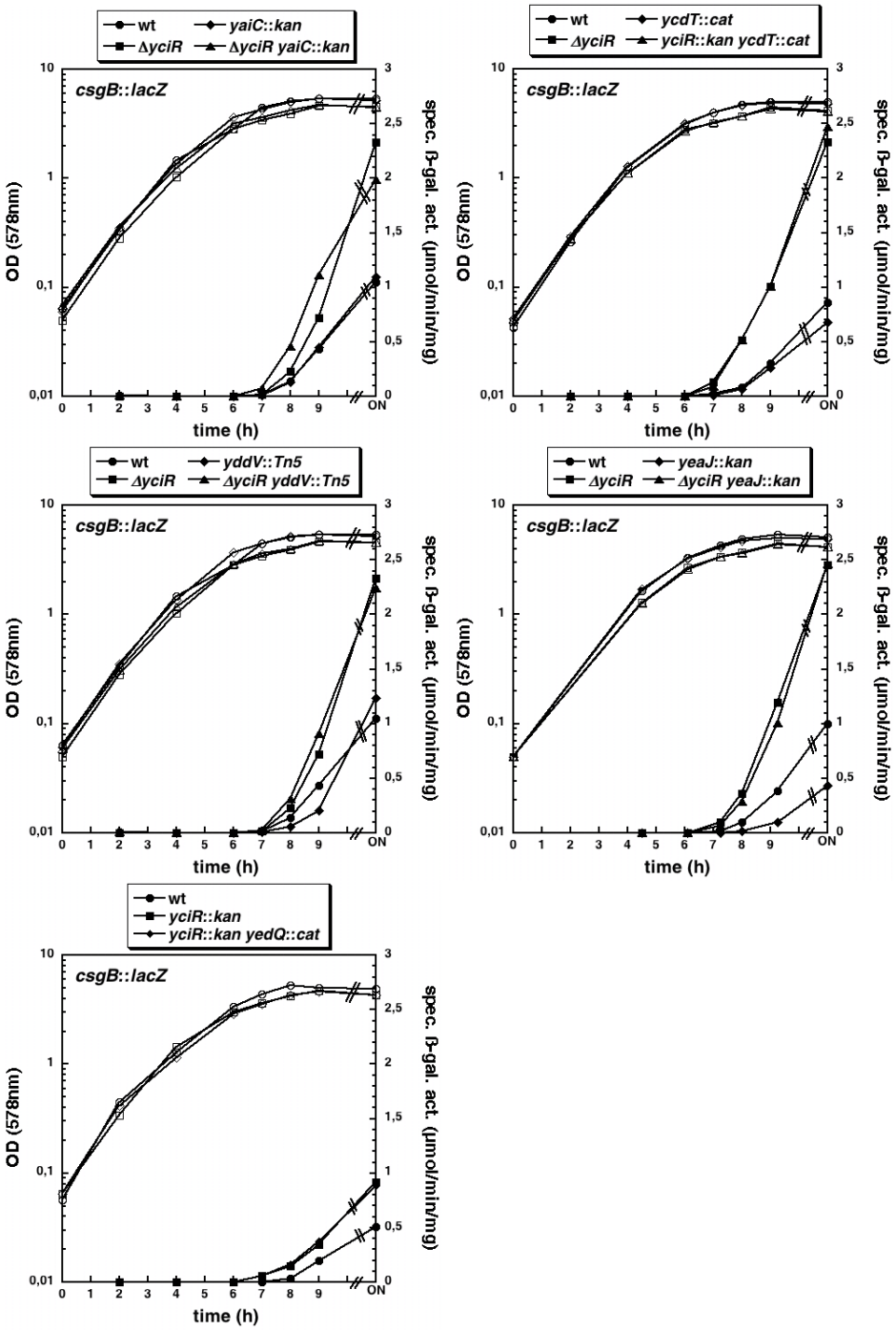

Fig. 4.39: Mutations in several GGDEF proteins do not reduce curli fimbriae expression in the *yciR* mutant. Expression of a single copy chromosomal *csgB::lacZ* fusion in MC4100 wild-type (wt) cells and derivatives carrying single and double mutations in *yciR* and the indicated GGDEF genes. Cells were grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

4.3.1.4 H-NS influences curli fimbriae expression through its influence on *yciR* and *ydaM* expression

Although H-NS was found to exert a repressing effect on *csgD* transcription in *Salmonella* that is based on binding to the region upstream of the *csgD* promoter region, the net effect of

H-NS-mediated regulation of *csgD* transcription was positive due to an uncharacterized activating role of H-NS (Gerstel et al. 2003). Consistently, data obtained by the Hengge group had shown that H-NS also acts as an activator of *csgD* transcription in *E. coli*. Moreover, these data had indicated that H-NS may mediate its positive effect on *csgD* transcription through regulation of YdaM, since the influence of YdaM and YciR on *csgD* expression almost disappeared in the *hns* mutant background and, conversely, introduction of a *hns* mutation did not lead to further reduction of *csgD* transcription in a *ydaM* mutant background (Weber et al. 2006).

Thus, the influence of H-NS on expression of a *ydaM::lacZ* fusion was tested here. Indeed, *ydaM::lacZ* expression was altered in the *hns* mutant, with higher expression levels during exponential growth, but lower expression during entry into stationary phase as compared to the wild-type (Fig. 4.40A). In contrast, expression of a *yciR::lacZ* fusion was elevated in the *hns* mutant in both exponential and stationary phase (Fig. 4.40B). Thus, in the wild-type, H-NS activates expression of the DGC YdaM and represses its antagonist YciR during entry into stationary phase. As this opposing effect on the two antagonistic proteins can be expected to result in an increase of YdaM-mediated DGC activity, it explains the activating role of H-NS in induction of *csgD*. H-NS establishes an expression profile of YdaM and YciR that allows for accumulation of c-di-GMP during entry into stationary phase, i.e. at the time-point that is relevant for curli fimbriae expression. Therefore, H-NS-mediated regulation of *yciR* and *ydaM* further emphasizes the importance of expression regulation in the coordination of DGC and PDE activities.

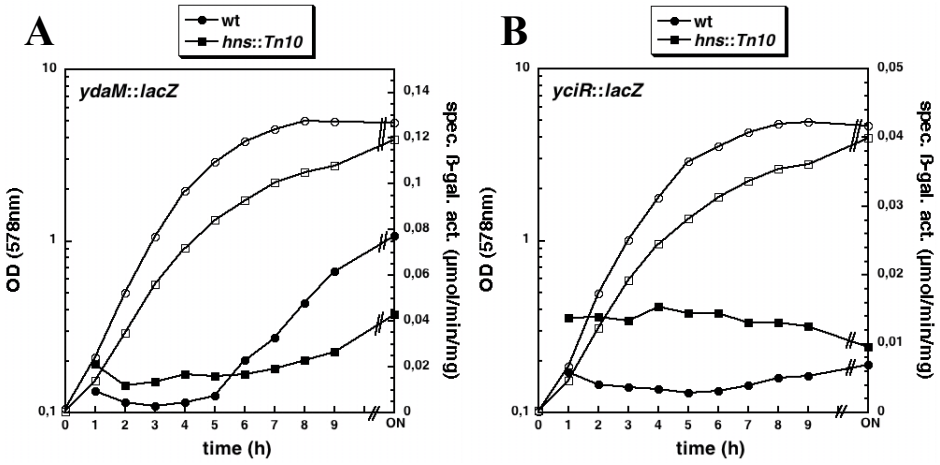

Fig. 4.40: H-NS influences expression of the YdaM/YciR curli control module. Expression of single copy chromosomal *lacZ* fusions to *ydaM* **(A)** and *yciR* **(B)** in MC4100 wild-type (wt) and *hns::Tn10* mutant derivatives. Cells were grown at 28°C in LB medium. OD_{578nm} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve and in overnight cultures (ON).

Together the data presented in this part of the thesis demonstrate that the molecular mechanisms underlying YdaM/YciR-mediated control of curli fimbriae expression go beyond simple synthesis and degradation of c-di-GMP. Moreover, DGCs other than YdaM may contribute to c-di-GMP-mediated induction of curli fimbriae synthesis. It was also revealed that tight control of *ydaM* and *yciR* expression plays an important role, firstly, because it contributes to establishing the right balance between the two regulators to allow for induction of curli fimbriae synthesis and secondly, by determining the functional context in which YciR acts. Expression control also seems to play a similar role in coordinating the activities of other EAL proteins.

4.3.2 MlrA binds to a sequence with similarity to a σ^S-dependent promoter that is located upstream of the *csgD* promoter

When this work was started, the exact binding site of the MerR-like regulator MlrA within the upstream regulatory region of the *csgD* promoter had remained unknown. Determining the MlrA binding site was not only of interest because it would provide additional information on the architecture of the intricate nucleoprotein complex established at the *csgD* promoter, but also because MlrA is a potential candidate for the missing effector mediating induction of *csgD* transcription by c-di-GMP (Hengge 2009a). Both the YdaM/YciR c-di-GMP control module and MlrA seem to exclusively regulate *csgD* transcription during entry into stationary phase (Weber et al. 2006; Weber 2007), thus raising the possibility that they act together in a microcompartmented fashion. Therefore identification of the binding site of MlrA may be an essential piece of information in the process of understanding the exact sequence of events contributing to the c-di-GMP-dependent activation of *csgD* transcription.

Earlier electrophoretic mobility shift experiments had given an approximate location of the MlrA binding site, suggesting that MlrA binds to a region more than 80 nucleotides upstream of the transcriptional start site, overlapping with a sequence that strongly resembles the consensus sequence of a σ^S-dependent promoter (Weber 2007). Therefore, footprint experiments with purified MlrA protein and two different DNA-fragments, one containing the known *csgD* promoter and the other one containing the sequence resembling a σ^S-dependent promoter, were performed. A single MlrA binding site was identified spanning nucleotides -141 to -114 relative to the transcriptional start site (Fig. 4.41A/C). Although this binding site was located slightly further upstream than predicted by the earlier EMSA studies, it still overlapped with the -35 element and part of the spacer of the putative σ^S-dependent promoter.

No additional MlrA binding sites could be identified in the region containing the known *csgD* promoter (Fig. 4.41B). Since primer extension experiments had not identified any transcriptional activity starting from the putative σ^S-dependent promoter (Weber 2007) under conditions promoting curli fimbriae expression, the relevance of this sequence in *csgD* transcription remains unclear.

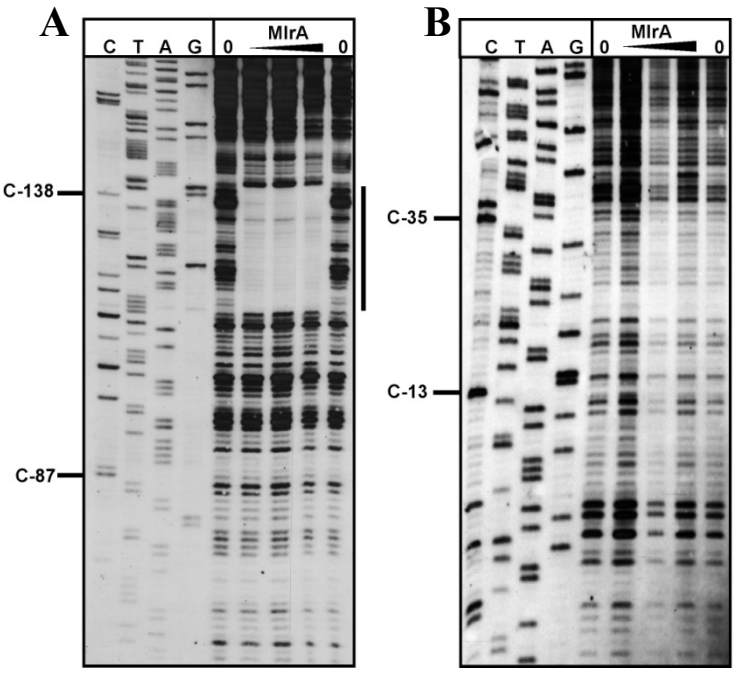

Fig. 4.41: MlrA binds to a sequence upstream of the *csgD* promoter that shows similarity to a σ^S-dependent promoter. Non-radioactive DNaseI footprint analysis, using purified MlrA and Digoxigenin-labelled DNA fragments containing either a region upstream of the known *csgD* promoter that also includes the sequence resembling a σ^S-dependent promoter **(A)** or a region containing the known *csgD* promoter **(B)**. The MlrA-binding site identified in (A) is indicated by a bar and was mapped to the upstream regulatory region of *csgD*, where it is marked by boxes **(C)**. The transcriptional start site for *csgD* (indicated by a red, underlined letter) had been determined before (the start site indicated here was identified by Harald Weber (Weber 2007) and differs by one nucleotide from the transcriptional start site published before (Hammar et al. 1995)). -10 and -35 elements of the *csgD* promoter and the putative σ^S-dependent promoter are indicated by red and blue italic letters, respectively. The sequencing reactions were performed with the same labelled primers used for the footprint experiments. Numbers indicate positions relative to the transcriptional start site.

5. Discussion

5.1 The lifestyle-switch from motility to adhesion is coordinated by multiple levels of mutual inhibition of the two cascades controlling curli fimbriae expression and flagellar motility

The switch between the motile-planktonic lifestyle and the sedentary-adhesive lifestyle that has been investigated in this work, is not only characteristic of the initial stages of biofilm formation, but is also part of an even more global transition. In environments providing limiting amounts of nutrients, bacteria display a foraging lifestyle that is characterized by increased motility to search out for more beneficial conditions and up-regulation of systems enabling efficient nutrient scavenging (Ferenci 2001; Zhao et al. 2007). If nutrient depletion continues, bacteria transition into the stationary phase lifestyle, in which they become resistant to many stresses, alter metabolic activities to ensure maintenance rather than growth and induce the expression of adhesive surface structures (Hengge 2010a). In this work, mutual exclusion of motility and the general stress response, part of which is curli fimbriae expression, was shown. This mutual exclusion does not only seem to follow the logic of an apparent interference of adhesion to surfaces with flagella-driven motility, but also reflects a more fundamental principle stating that high levels of stress resistance are established at the expense of rapid growth.

This principle is not restricted to the bacterial kingdom. A recent publication demonstrated that the *Arabidopsis thaliana* gene *ACD6* influences both the extent of vegetative growth and the resistance against pathogens and herbivorial predators (Todesco et al. 2010). Some strains carry a hyperactive allele of this gene that confers increased resistance against infection but at the same time slows down leave production and compromises leave biomass. Thus, fast growth is generally incompatible with increased resistance against adverse conditions and while both rapid proliferation and stress resistance convey advantages under specific conditions, this is at the expense of reduced fitness under other conditions. In *Arabidopsis thaliana* this seems to be compensated for by the co-occurrence of functionally distinct alleles of *ACD6* amongst global and local populations. This results in the simultaneous presence of plants that are able to produce substantial biomass in the absence of pathogens but are more susceptible to infection, and plants that produce less biomass but are able to withstand pathogenic attacks (Todesco et al. 2010). In contrast, bacteria deal with the

predicament of balancing rapid growth versus survival under adverse conditions by employing regulatory systems that allow switching between the two lifestyles according to environmental conditions. A highly complex, interlinked and fine-tuned signal transduction network ensures switching only under very specific conditions that are threatening enough to justify the reallocation of limited resources to survival mechanisms at the expense of reduced proliferation. The regulatory network controlling the transition between motility and adhesion represents an example for such a sophisticated system.

5.1.1 An overview

The analyses presented in part 4.1 of this work together with results obtained by Gisela Becker (Pesavento et al. 2008) identified several layers of crosstalk between the cascades controlling curli fimbriae expression and flagellar motility (summarized in Fig. 5.1) that combine to establish mutual exclusion of the two systems and form the basis for the switch between motility and adhesion: In post-exponential phase motility peaks due to maximal flagellar gene expression and concomitantly several members of the flagellar regulon contribute to repression of curli fimbriae expression for as long as flagellar gene expression continues. The flagellar regulator FliZ interferes with the activity of σ^S, which begins to accumulate in the cell but due to FliZ is unable to induce curli fimbriae expression, a process under multiple feedforward control of σ^S. At the same time, σ^{70} and σ^{28} successfully compete with σ^S for limiting amounts of RNAP core enzyme. σ^{28} also induces expression of the phospodiesterase YhjH. YhjH keeps cellular c-di-GMP levels low and thereby not only ensures maximal motility, but also interferes with induction of the curli regulator CsgD.

However, when cells enter stationary phase, flagellar gene expression and in consequence expression of FliZ, σ^{28} and YhjH ceases. Decreasing levels of FliZ and increasing $E\sigma^S$ formation allow σ^S to access its target promoters and rising σ^S levels increase competition for RNAP core enzyme to the disadvantage of σ^{70} and σ^{28}, thereby most likely contributing to the shut-down of flagellar gene expression. At the same time σ^S-mediated induction of YegE (and to a lower extent induction of YedQ) leads to increased c-di-GMP synthesis, finally overcoming the negative effect of YhjH. The expression of *yhjH* (like the expression of all flagellar genes) is precisely turned off during transition into stationary phase due to ClpXP-mediated proteolysis of the flagellar master regulator $FlhD_4C_2$, thereby allowing for a decline in phosphodiesterase activity. The resulting elevated c-di-GMP level does not only inhibit activity of the flagella via YcgR, but also contributes to stimulation of

csgD expression. In addition to YegE/YedQ, induction of *csgD* transcription essentially requires c-di-GMP synthesized by the σ^S-dependent diguanylate cyclase YdaM.

In summary, this complex regulatory system only allows for induction of curli fimbriae expression after flagellar gene expression and flagellar activity have been down-regulated. Figure 5.1 summarizes the communication network between the cascades controlling motility and curli fimbriae expression and also includes further regulatory influences that, together with other details of this multifaceted switch control, will be discussed in the following sections.

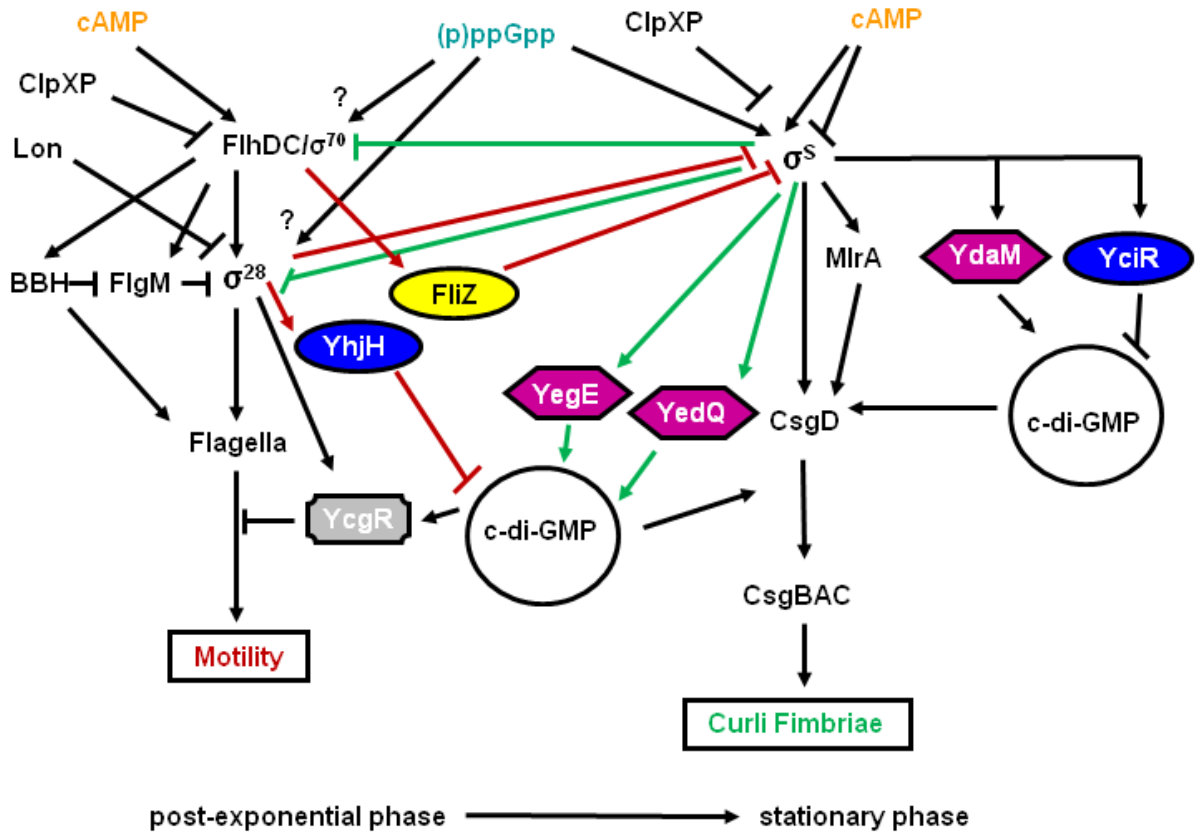

Fig. 5.1: Model summarizing the regulatory links between the cascades controlling flagellar motility and curli fimbriae expression in *Escherichia coli*. The model shows the different levels of communication between the two cascades and also includes potential and demonstrated regulatory influences of proteolysis, cAMP and (p)ppGpp that affect the regulatory links between the cascades. Diguanylate cyclases are coloured pink, phosphodiesterases blue. Inhibitory effects of the motility system onto the σ^S/curli system are denoted in red and σ^S-mediated effects contributing to down-regulation of motility are shown in green. BBH denotes the hook-basal body complex. Question marks indicate the not yet clarified influence of (p)ppGpp on motility. For details refer to the main text.

5.1.2 The role of sigma factor competition in the switch between the two lifestyles

The crosstalk between the motility and curli regulatory cascades affects all levels of control i.e. transcription, proteolysis and enzymatic activities. On the level of transcription, sigma factor competition between the involved sigma factors σ^{70}, σ^{S} and σ^{28} for RNAP core enzyme plays a major role. Sigma factor competition for limiting amounts of RNAP core enzyme has been suggested to directly reflect the trade-off between proliferation and stress resistance at the molecular level (Nyström 2004). Consistent with this, competition between σ^{70}/σ^{28}, which control features promoting growth and foraging behaviour, and σ^{S}, which is dedicated to induce stress resistance and adhesion was shown here to essentially contribute to the inverse coordination of motility and adhesion in *E. coli*.

σ^{S} contributes to shutting down flagellar gene expression and thereby also to down-regulation of its antagonist FliZ (Fig. 4.6B and results obtained by other members of the Hengge group, (Pesavento et al. 2008)). Confirming these results, higher stationary-phase expression of flagellar genes in a *rpoS* mutant has also been reported for a different *E. coli* strain grown in both rich and minimal medium (Patten et al. 2004; Dong and Schellhorn 2009). Since the expression of σ^{70}-dependent genes is known to be induced in the absence of σ^{S}, which seems to be due to increased binding of σ^{70} to RNAP core enzyme (Farewell et al. 1998), this is likely to be the basis for prolonged σ^{70}/σ^{28}-driven flagellar gene expression in the *rpoS* mutant. Interestingly, a recent publication revealed that competition between σ^{S} and σ^{N}, which belongs to the σ^{54} family of sigma factors, plays an important role in the regulation of motility (Dong et al. 2011). Under certain conditions motility was shown to depend on σ^{N}. Similar to our observations, motility and flagellar gene expression were enhanced in a *rpoS* mutant under these conditions and this required the presence of σ^{N} (Dong et al. 2011). Thus sigma factor competition seems to generally play an important role in the regulation of motility and a more global role in the coordination of cellular responses directed by the competing sigma factors. In addition to the observed coordination of motility with the general stress response, this can for example imply inverse regulation of the σ^{S}-controlled general stress response with σ^{N}-controlled utilization of nitrogen sources (Dong et al. 2011).

It is interesting to note that the activity of the extracytoplasmic stress sigma factor σ^{E} also increases during entry into stationary phase (Costanzo and Ades 2006), thus showing that σ^{E} is also part of the ensemble of competing sigma factors during this transition.

Moreover, σ^{28} was shown here to contribute to repression of curli fimbriae expression in the presence of continuous induction of the flagellar gene regulon (Fig. 4.6A). The role of σ^{28} in this repression could simply lie in induction of the class III gene *yhjH*, because prolonged expression of YhjH interferes with curli fimbriae expression due to a need for down-regulation of its phosphodiesterase activity for induction of CsgD expression (see 4.1.5). However, earlier induction of *mlrA* expression in the *fliA* mutant (Fig. 4.5D) strongly supports that the influence of σ^{28} is not restricted to induction of YhjH, since *mlrA* expression is not influenced by YhjH (shown by Gisela Becker in (Pesavento et al. 2008)). Hence, competition between σ^{28} and σ^{S} in post-exponential phase, when σ^{28} levels are high, most likely interferes with induction of curli fimbriae expression by limiting access of σ^{S} to RNAP core enzyme. Thereby σ^{28} acts in concert with FliZ to block induction of the σ^{S} regulon for as long as flagellar gene expression continues. Thus, sigma factor competition together with FliZ-mediated inhibition of σ^{S} activity establishes a basic level of control that inversely coordinates flagellar gene expression with the general stress response. On top of this, the c-di-GMP-based control mechanism involving YhjH, YegE and YedQ adds another more specific level of inverse control that affects motility, but apparently only one module within the general stress response, i.e. curli fimbriae expression.

The signalling molecule (p)ppGpp, which is induced by signals resulting from nutrient limitation and different stresses, is known to influence sigma factor competition, hence regulating it in response to environmental conditions (Jishage et al. 2002; Nyström 2004; Gummesson et al. 2009). By increasing the relative competitiveness of alternative sigma factors for RNAP core enzyme at the expense of σ^{70}, (p)ppGpp also positively influences σ^{S} activity and, in addition, (p)ppGpp has a positive effect on *rpoS* transcription (Lange et al. 1995). Consistently, induction of the general stress response was shown to depend on (p)ppGpp (Traxler et al. 2006; Traxler et al. 2008). In contrast, inconsistent results have been obtained concerning the role of (p)ppGpp in motility regulation, indicating that the nature of its effect on motility, although mostly positive, might be highly dependent on environmental conditions (Magnusson et al. 2007; Durfee et al. 2008; Aberg et al. 2009; Gummesson et al. 2009). Together, participation of (p)ppGpp in the regulation of motility, sigma factor competition and σ^{S} expression hints at an important role in the inverse coordination of motility and adhesion, and growth phase-dependent alterations in (p)ppGpp concentrations can be expected to contribute to triggering the switch between the two lifestyles. This hypothesis, however, requires further experimental verification.

Moreover, cAMP is known to have an influence on the expression of σ^S and on the expression of the flagellar master regulator operon *flhDC* (and therefore also on the expression of σ^{28}), providing another regulatory link that influences the levels of and balance between the sigma factors involved in motility and curli fimbriae regulation in response to the nutrient status of the environment. While cAMP-CRP activates expression of the *flhDC* operon (Soutourina et al. 1999), the role of cAMP in transcription of *rpoS* depends on the growth-phase, with cAMP repressing *rpoS* transcription during exponential growth and activating it during entry into stationary phase (Hengge-Aronis 2002a; Mika and Hengge 2005). Thus, cAMP can be expected to inversely control motility and σ^S-dependent gene expression during exponential growth, when motility is predominant, while it activates σ^S expression in stationary phase. Interestingly, motility gene expression was shown to be generally up-regulated in poor quality carbon sources in a cAMP-CRP-dependent manner, but expression of the filament protein FliC was not affected (Zhao et al. 2007). Based on these results, the authors suggested that cAMP-CRP triggers increased motility to allow cells to actively search out for better conditions once carbon source quality declines, but that this increase in motility involves increased flagellar operation rather than increased synthesis of new flagella, thus optimizing the investment of limited energy resources. However, if conditions further deteriorate, motility declines, indicating that cells are forced to employ a different strategy to ensure maintenance under the threat of vanishing energy resources (Zhao et al. 2007). Thus, it will be interesting to examine which role cAMP plays in timing the switch between motility and curli fimbriae expression in response to the nutritional status of the environment.

5.1.3 The role of FliZ and MlrA as timing devices

Repression of σ^S activity by the flagellar protein FliZ represents another level of transcriptional control. FliZ was identified here as a potent inhibitor of σ^S-dependent gene expression and by delaying the induction of σ^S-dependent genes, FliZ transiently gives motility priority over the general stress response for as long as flagellar gene expression continues. σ^S is already accumulating in the cells in post-exponential phase but is unable to induce its full regulon since many of the σ^S-dependent promoters are blocked by FliZ. This inhibition is only alleviated once flagellar gene expression begins to cease and FliZ levels start to decline, while $E\sigma^S$ levels concomitantly rise upon entry into stationary phase.

As indicated by the microarray analysis aimed at identifying the FliZ regulon, not all genes of the σ^S regulon are subject to inhibition by FliZ. In this respect, it is interesting to note

the strong overlap between negatively FliZ-regulated genes and genes activated by Crl (Tab. 4.1). Crl positively affects σ^S activity by supporting σ^S in its competition for RNAP core enzyme (Typas et al. 2007a). Both FliZ and Crl exert their strongest influence on genes like *csgB* and *gadB*, which are under multiple feedforward control of σ^S, and on genes like *mlrA*, which are controlled by rather weak promoters. The affinity of promoters plays an important role in determining how susceptible the expression of a gene is to sigma factor competition and to regulation by other factors influencing the activity of the respective RNAP holoenzyme transcribing the gene. Using mathematical modelling it was predicted that weak promoters are more strongly affected by alterations in sigma factor competition than high affinity promoters (Grigorova et al. 2006). This model was supported experimentally by a comparison of the effects of DksA and (p)ppGpp on a set of σ^{54}-dependent promoters with varying affinity, which directly demonstrated that the low-affinity promoters were significantly more sensitive to the loss of these two factors (Bernardo et al. 2006). This was attributed to the (p)ppGpp and DksA-mediated increases in free core enzyme levels that is a consequence of the down-regulation of rRNA synthesis by these regulators. The consequent increase in $E\sigma^{54}$ holoenzyme levels under conditions that cause an increase in (p)ppGpp levels has more pronounced effects at low-affinity promoters than at high-affinity promoters, since the latter can be expected to be saturated over a wider range of holoenzyme concentrations (Bernardo et al. 2006).

These results can most likely be transferred to other alternative sigma factors and are consistent with the observations reported here regarding expression of the *mlrA* promoter. Although affinity of the *mlrA* promoter has not been experimentally tested, analysis of its sequence shows the absence of activity enhancing features such as G(-14) or a -35 region (Typas et al. 2007b), which is also reflected in the low *mlrA* expression level (compare e.g. expression of *mlrA* in Fig. 4.3A with expression of the σ^S-dependent genes *osmY* and *gadB* in Fig. 4.4). This strongly indicates that the *mlrA* promoter is a weak, low-affinity promoter and thus an ideal target for regulation by factors influencing levels and activities of RNAP containing σ^S, which was confirmed here using several mutants that alter $E\sigma^S$ levels and activities, including Crl and FliZ. Consistent with the important role that changes in holoenzyme composition play in lifestyle-transitions (see above), all these mutations modified the timing of *mlrA* induction (Fig. 4.5D). In the case of FliZ, stronger influences on low-affinity promoters may be due to an increased capability of FliZ to compete with $E\sigma^S$ for promoter binding at these promoters.

As MlrA is the latest regulator of the curli control cascade to be induced, induction of *mlrA* expression times curli fimbriae expression. It therefore is the major point at which regulatory influences that delay (FliZ, σ^{28}) or promote earlier induction (Crl, Rsd) of $E\sigma^S$ activity are integrated into curli regulation.

The influence of FliZ is highest in post-exponential phase, when σ^S starts to accumulate. Similarly, the positive influence of Crl on incorporation of σ^S into RNAP holoenzyme is also highest at low levels of σ^S (Typas et al. 2007a). Later induction of *mlrA* in a *crl* mutant, but earlier induction in the *fliZ* mutant (Fig. 4.5D) therefore further supports that Crl and FliZ exert functionally antagonistic roles by promoting and delaying $E\sigma^S$ activity at low σ^S levels, respectively.

The role of FliZ as a timing device is not restricted to the expression of curli fimbriae. As demonstrated in section 4.2.4.3 FliZ also contributes to the temporal sequestration of a phosphodiestrase, YciR, which thereby becomes functionally restricted to regulation of curli fimbriae synthesis. In addition, FliZ participates in negative feedback control of motility gene expression (Fig. 4.28D). While the role of FliZ in these contexts will be discussed in more detail below, it further corroborates the important role of FliZ in coordinating the precise expression of regulators influencing curli fimbriae expression and motility.

5.1.4 Down-regulation of the flagellar system is a prerequisite for switching from motility to adhesion

With the flagellar system encoding three regulators interfering with σ^S activity and/or induction of curli fimbriae expression, i.e. FliZ, YhjH and σ^{28}, down-regulation of flagellar gene expression and inactivation of already existing gene products is a prerequisite not only for the transition to the adhesive lifestyle under appropriate conditions (e.g. low temperature), but also for induction of the general stress response. Thus, shutting down the flagellar system under dwindling nutrient conditions does not only save energy, but is a necessary step on the way to induction of maintenance metabolism, stress resistance and adhesion.

The fact that FliZ also exerts its repressing influence at 37°C (Fig. 4.3D and Fig. 4.5B) indicates that even under conditions that do not promote curli fimbriae expression, FliZ still interferes with σ^S-dependent gene expression and together with σ^{28}/σ^S competition for RNAP core enzyme coordinates inverse regulation of motility with σ^S-dependent gene expression. This again suggests that these regulators are part of a more global mechanism directing allocation of limiting resources in the trade-off between foraging/proliferation and

survival/maintenance and emphasizes the need for down-regulation of the flagellar system to allow for induction of the general stress response.

Consistent with the strong requirement for elimination of all inhibitory factors, the flagellar system is efficiently and very precisely down-regulated on all possible levels when cells enter stationary phase: Flagellar gene expression ceases and ClpXP-mediated degradation of the master regulator $FlhD_4C_2$ helps to relay stop of class I gene expression to class II and III expression. At the same time, σ^S-dependent induction of c-di-GMP production interferes with the function of already existing flagella. The mechanism mediating c-di-GMP-dependent inhibition of flagellar movement has recently been further elucidated by three publications demonstrating that c-di-GMP binding to the effector protein YcgR inhibits flagellar function by promoting interaction of YcgR with flagellar motor proteins (Boehm et al. 2010; Fang and Gomelsky 2010; Paul et al. 2010). However, controversy remains over which of the motor proteins are involved in YcgR binding and about the direct molecular consequences of this interaction.

The exact mechanisms mediating repression of flagellar gene expression in stationary phase remain unknown, but sigma factor competition likely plays an important role, as discussed above. Whether the contribution of σ^S to shutting down flagellar gene expression is restricted to its role in sigma factor competition or if e.g. another σ^S-dependent regulator is more directly involved in this repression, was not further clarified here.

Since both *fliZ* mRNA and FliZ protein levels drastically drop upon entry into stationary phase (Fig. 4.7), it seemed unlikely that competition between σ^S and the sigma factors involved in flagellar gene expression is the only mechanism involved in this repression. However, none of the regulators tested here, which had been selected due to a link to flagellar gene expression and/or their strong induction in stationary phase, contributed to shutting down FliZ. This does not rule out the possibility that one or several of these regulators participate in the down-regulation of FliZ, or flagellar gene expression in general, under other conditions. For instance, the σ^S-dependent repressor NsrR might not be active under the conditions tested here, but might be employed to specifically coordinate motility and σ^S-dependent gene expression under other conditions, e.g. in the minimal medium used when NsrR-mediated regulation of flagellar genes was identified (Partridge et al. 2009). Similarly, FliY was recently shown to be strongly induced in the presence of hydrogen peroxide and to be required for the defence against this stressor (Ohtsu et al. 2010). Thus, FliY may be involved in regulation of motility in response to hydrogen peroxide. By employing such condition-specific regulators cells may fine-tune the motility and general

stress response systems in response to very specific environmental conditions to yield a cellular response that is best-fitted to the respective situation. In this context, it would e.g. be interesting to see how the presence of nitric oxide, which is known to inactivate NsrR and in turn activates the expression of *fliA* (Partridge et al. 2009), affects expression of genes belonging to the σ^S regulon, and how the presence of hydrogen peroxide affects motility and curli fimbriae expression.

Another intriguing question is how FliZ-mediated repression of σ^S-dependent gene expression is eliminated when cells are exposed to stresses during exponential growth, when flagellar gene expression is high. Under these conditions flagellar gene expression can be expected to be quickly shut-down, due to both a rapid increase in σ^S levels affecting sigma factor competition and induction of specific repressors, e.g. OmpR, which represses *flhDC* expression and is induced in response to high osmolarity. Quick adaptation to the stress through induction of σ^S-dependent genes may, however, also require inactivation of FliZ which is present at high levels in exponential phase. This could be mediated by interaction of FliZ with another regulator that inhibits its activity, e.g. by blocking FliZ binding to promoter DNA. Alternatively, exposure to stress during exponential growth may result in FliZ proteolysis. As mentioned above, analysis of FliZ-proteolysis *in vivo* is impossible, due to the presence of multiple proteolysis targets in the cascades controlling motility and curli fimbriae expression. Proteolysis of FliZ will therefore be examined in future experiments using an *in vitro* proteolysis system established in the Hengge group. It should be noted in this context that a recent publication suggests that FliZ might be a substrate of the Lon-protease (Chubiz et al. 2010). Consistent with this, presence of the pFliZ plasmid used here strongly inferred with growth of a *lon* mutant (data not shown). A similar growth defect was also observed in wild-type cells when FliZ was over-produced (data not shown), suggesting that FliZ accumulates in the *lon* mutant. Thus, the Lon protease seems to be a good candidate for mediating FliZ degradation and should be included in the *in vitro* proteolysis tests.

In contrast, the data presented here indicate that ClpXP-mediated proteolysis of the master regulator $FlhD_4C_2$ plays an essential role in the precise down-regulation of the phospho-diesterase YhjH upon entry into stationary phase. A *clpP* mutant is unable to induce curli fimbriae expression, which provides the first clear-cut phenotype of a *clpP* mutation in *E. coli*. Repression of curli fimbriae expression was suppressed by introduction of a mutation in *yhjH* (Fig. 4.13C). Since experiments performed by other members of the Hengge group showed that YhjH is unlikely to be subject to proteolysis under the conditions used here

(Pesavento et al. 2008), prolonged expression of *yhjH* in the *clpP* mutant seems to be responsible for the failure of the *clpP* mutant to induce curli fimbriae expression (Fig. 4.14). Down-regulation of YhjH is necessary to allow for accumulation of c-di-GMP that is required to induce CsgD expression and to inhibit the activity of the already existing flagella, which may be required to allow attachment to surfaces and to other cells. $FlhD_4C_2$ proteolysis allows class II and III gene expression to quickly respond to the shut-down of *flhDC* expression through removal of the already existing $FlhD_4C_2$. Proteolysis is necessary to eliminate $FlhD_4C_2$ since already synthesized proteins will no longer be rapidly and sufficiently diluted by cell division in stationary phase cells. Consistently, the second major inducer of flagellar genes, σ^{28} is also subject to proteolysis, in this case mediated by the Lon protease (Barembruch and Hengge 2007). The requirement for $FlhD_4C_2$-degradation to allow for induction of curli fimbriae synthesis finally provides a physiological rationale for ClpXP-mediated proteolysis of the flagellar master regulator, which had been described years ago without elucidating its function (Tomoyasu et al. 2003).

Interestingly, a study published in parallel to results presented here showed that ClpXP-mediated degradation of $FlhD_4C_2$ is required for shutting down the expression of *Salmonella* pathogenicity island I (SPI1) genes, once systemic infection has been established by the pathogen (Kage et al. 2008). Amongst others, the SPI1 type III secretion system has been shown to be involved in induction of macrophage apoptosis (Hersh et al. 1999). Since *Salmonella* uses macrophages as vectors for dissemination throughout the host after systemic infection has been established, macrophage apoptosis needs to be down-regulated at this stage. ClpXP-mediated degradation of $FlhD_4C_2$ contributes to shutting down SPI1 gene expression through its effect on the expression of FliZ, which acts as an activator of SPI1 gene expression (Kage et al. 2008). These results do not only provide an additional physiological role for ClpXP-mediated proteolysis of $FlhD_4C_2$, but also hint at a potential role of FliZ in the regulation of another lifestyle transition observed in pathogenic bacteria, i.e. the transition from invasion to dissemination and systemic infection. The role of FliZ in virulence will also be discussed below (see 5.2.4).

It is interesting to note that a mutation in *fliZ* suppressed the repressing effect that ectopic *flhDC* expression exerts on curli fimbriae expression (Fig. 4.2B), but did not alleviate repression of curli fimbriae expression in the *clpP* mutant (Fig. 4.11), although curli repression seems to be due to elevated $FlhD_4C_2$ levels during entry into stationary phase in both cases. This discrepancy can most likely be explained by the fact that the flagellar sigma factor σ^{28}, besides being degraded by Lon protease, is also a minor substrate of the ClpP

protease and therefore stabilized in the *clpP* mutant (Barembruch and Hengge 2007). Thus, class III gene expression in this mutant will not only be prolonged into stationary phase because the effect of $FlhD_4C_2$ stabilization on class II gene expression is relayed to class III gene expression (which is the same in the strain ectopically expressing *flhDC*), but is more efficiently induced by stabilization of σ^{28}. Thus a mutation in *fliZ* alone is not sufficient to alleviate repression of curli fimbriae expression due to strong expression of the class III gene product and curli inhibitor YhjH in this strain.

5.2 FliZ is a novel type of σ^S antagonist

5.2.1 FliZ is a novel type of sigma factor antagonist that uses sigma factor mimicry to bind to and most likely occlude σ^S-dependent promoters

Considering its general effect on σ^S-dependent gene expression, FliZ seemed like a *bona fide* candidate for an anti-sigma factor. In contrast to typical anti-sigma factors, however, FliZ does not directly interact with σ^S (Fig. 4.16) but binds to and therefore most likely occludes the -10 elements of σ^S-dependent promoters. A mechanism involving DNA binding seemed unlikely at first, since σ^S and the vegetative sigma factor σ^{70} recognize almost identical promoter sequences, thus raising the puzzling question of how FliZ is able to discriminate between these very similar promoters.

FliZ solves this problem by structural mimicry of the promoter recognition element of σ^S. A putative alpha helix in FliZ shows striking sequence similarity to the first alpha helix in domain 3 of σ^S, which mediates specific binding to extended -10 elements. In particular, K173 in the promoter recognition element of σ^S and the corresponding R108 in the putative alpha helix of FliZ are both involved in promoter binding. A single amino acid exchange at position R108 in FliZ strongly reduced its ability to bind to promoter DNA *in vitro* and also alleviated repression of a target gene *in vivo* (Fig. 4.24 and Fig. 4.25), indicating that this amino acid plays a central role in promoter recognition by FliZ. An analogous role to the one assumed by K173 of σ^S, which directly binds to the TC(-14/-13) promoter element, was further supported by the finding that mutations in this sequence element also reduced promoter binding by FliZ (Fig. 4.23). Binding of the TC(-14/-13) element by the positively charged K173 residue in σ^S plays an important role in establishing σ^S selectivity of promoters, since the oppositely charged glutamate residue at the corresponding position in σ^{70} conveys a different preference for the nucleotides at these positions in the promoter (Becker and Hengge-Aronis 2001).

Together these mutational analyses strongly support that FliZ employs the positively charged R108 residue, which is situated in a structural element with strong similarity to the promoter recognition element in σ^S, to specifically recognize a hallmark of σ^S-dependent promoters. Moreover, T(-12) and T(-7), which are part of the core -10 hexamer recognized by sigma factors, also contribute to binding of FliZ. Thus, σ^S and FliZ employ very similar mechanisms for recognizing and binding to the extended -10 region.

However, the whole genome transcription profiling experiments revealed that not all σ^S-dependent genes are also FliZ-controlled, indicating that additional features, which contribute to optimal binding of either σ^S or FliZ, may not be identical. The -35 element, which is contacted by a different domain of σ^S, might constitute an example of such an element that might not play a role in FliZ binding. Furthermore, numerous additional promoter features, such as suboptimal spacer length or the combination of a -35 element with a distal UP-element half-site, are known to contribute to selective promoter recognition by σ^S, even in the presence of -10 regions that are similar to those of vegetative promoters (Typas et al. 2007b). However, since not all FliZ-dependent promoters tested here contain the C(-13), some of these additional σ^S selectivity inducing features may also play a role in the specific recognition of σ^S-dependent promoters by FliZ.

Conversely, FliZ binding may require sequence or structural features not recognized by σ^S. This is supported by the binding of FliZ to the operator site downstream of the *flhDC* promoter that does not seem to be part of a functional promoter. It is tempting to speculate that this operator site represents the remnants of a σ^S-dependent promoter that has specifically lost features important for σ^S binding, but retained features essential for recognition by FliZ. Moreover, many promoters, such as the *flhDC* and *csgD* promoters, are targeted by multiple regulators which will not only influence RNAP binding, but also recognition by FliZ. A different DNA conformation, e.g. differences in bending of promoter DNA, in the absence of these regulators may explain the ambiguous binding pattern observed in ELISAs with *csgD* promoter DNA (Fig. 4.18B). Thus, future experiments should aim at elucidating the differences in promoter recognition between σ^S and FliZ and also include the role that additional regulators play under different growth conditions.

How exactly does FliZ binding to promoter DNA interfere with $E\sigma^S$ activity? The most likely explanation is that occlusion of binding sites prevents $E\sigma^S$ from binding in the presence of FliZ. Unfortunately, it was impossible to establish assay conditions that allowed the direct confirmation of competitive promoter binding, since stability and DNA binding by $E\sigma^S$ and FliZ require highly different assay conditions. Thus, although it seems unlikely due

to the overlap in important promoter sequence determinants for binding of $E\sigma^S$ and FliZ, simultaneous binding of both regulators cannot be ruled out at this stage. In this case, FliZ might mediate its repressing effect by changing the local DNA conformation in a way that allows $E\sigma^S$ binding but prevents successful transcription initiation, e.g. by interfering with open complex formation or downstream steps. For λ integrase it was shown that DNA binding of the core-binding domain stimulates activity of the catalytic domain, most likely through introduction of a conformational change in the DNA substrate that renders it more suitable for catalysis (Subramaniam et al. 2007). Since the alpha helix involved in DNA binding of FliZ is part of a region that shows partial similarity to the core-binding domain of phage integrase family proteins (also see below), alterations in the local DNA conformation upon FliZ binding seem possible. Clarification of these details concerning promoter binding of FliZ in the presence and absence of $E\sigma^S$ therefore requires further biochemical and/or crystallographic data.

Interestingly, a recent report revealed a different mechanism of sigma factor mimicry, employed by the response regulator PhyR to induce the general stress response in the alpha-proteobacterium *Methylobacterium extorquens* (Francez-Charlot et al. 2009). PhyR contains an amino terminal sigma factor-like domain in which determinants for promoter binding are degenerate or lost. Upon phosphorylation at its carboxy terminal receiver domain, the sigma factor-like domain of PhyR interacts with an anti-sigma factor, thereby releasing a sigma factor, which results in transcription of genes of the general stress response. Thus, similar to FliZ, PhyR uses a domain with similarity to a sigma factor to block binding of the "real" sigma factor to a site specifically recognized by the latter. While FliZ seems to occlude DNA sites recognized by σ^S, PhyR blocks the binding site on an anti-sigma factor, resulting in opposing effects on sigma factor activity. By mimicking parts of the sigma factor relevant for the respective function targeted by FliZ and PhyR, both proteins therefore act as "pseudo-sigma factors" that compete with the "real" sigma factors for binding sites on interaction partners.

Molecular mimicry is also part of the virulence mechanism of the intracellular pathogen *Legionella pneumophila*. The causative agent of Legionnaires´ disease encodes a large number of eukaryotic-like proteins, including proteins that exhibit high similarity to eukaryotic proteins and proteins carrying motifs implicated in protein-protein interactions in eukaryotic cells (Brüggemann et al. 2006). These eukaryotic-like proteins have been suggested as potential virulence factors that interfere with host cell processes by mimicking

their eukaryotic counterparts. A recent publication showed that one of these proteins contains an F-box domain, which is present in conserved eukaryotic proteins involved in recruiting polyubiquitinated proteins (Price et al. 2009). Through functional and structural mimicry this *Legionella* protein hijacks the conserved eukaryotic polyubiquitination machinery to modify the pathogen-containing vacuole in a way ensuring intracellular proliferation of the bacterium. Thus, molecular mimicry might be a mechanism more generally employed by bacteria to interfere with intrinsic as well as host cell processes and it is interesting to speculate about this form of mimicry from an evolutionary point of view. While some of the genes encoding eukaryotic-like proteins in *Legionella pneumophila* have been acquired through horizontal inter-domain gene transfer and fusion events between eukaryotic and bacterial gene fragments, others seem to originate from random mutational events that allowed bacterial genes to attain certain eukaryotic-like sequence motifs in the process of adaptation to life within the eukaryotic cell (Lurie-Weinberger et al. 2010). In FliZ, the C-terminal domain that includes the putative alpha helix involved in promoter recognition shows partial similarity to the core-binding domain present in proteins of the phage integrase family and the potential implications of these homologies will be discussed in the following section.

5.2.2 Similarities in DNA recognition by FliZ, domain 3 of sigma factors and phage integrase family members

Phage integrase family members catalyze site-specific recombination events e.g. during phage integration and excision or chromosome segregation. The N-terminal core-binding domains of these enzymes bind to so called core-type DNA sites, inverted repeats, which are part of the longer sites targeted by these proteins (Tirumalai et al. 1998).

An alignment of FliZ with the core-binding domains of several members of the phage integrase family for which structural information is available, revealed that the putative alpha helix in FliZ that mimics the extended -10 element recognition helix in σ^S corresponds to an alpha helix involved in DNA binding in phage integrase family members (Fig. 5.2) (Guo et al. 1997; Subramanya et al. 1997; Swalla et al. 2003; Biswas et al. 2005). The alignment shows that many residues strongly conserved in phage integrase family proteins are also present in FliZ. Interestingly, the predicted alpha helix in FliZ that contains residue R108 corresponds to one of a pair of orthogonally crossed alpha helices in the recombinase Cre, which make direct DNA contacts (Guo et al. 1997).

Moreover, the residue corresponding to E109 in FliZ plays a role in determining core-type DNA-binding specificity in λ integrase (Dorgai et al. 1995; Yagil et al. 1995). R108 and

the two neighbouring amino acids in FliZ are flanked by strongly conserved residues (Fig. 5.2 and (Swalla et al. 2003)). Swalla et al. (2003) suggested that variable regions within conserved structural elements may be involved in establishing the unique and enzyme-specific function of each core-binding domain. Thus, while the overall structure of the DNA-binding helix is maintained in FliZ, variable residues seem to be employed for recognition of σ^S-specific promoter elements.

By also including the first alpha helix of σ^S domain 3, the alignment shown in figure 5.2 indicates that the DNA-binding helices in FliZ and domain 3 of σ^S seem to both follow this pattern of conserved residues being interspersed with variable residues used for specific DNA sequence recognition. This suggests that FliZ and domain 3 of σ^S may use a similar mechanism of DNA interaction as proteins of the phage integrase family. This may indicate that domain 3 of sigma factors and the DNA binding domains of phage integrase family proteins are descendants of a common ancestor mechanism for DNA recognition. Thus, the molecular mimicry mechanism employed by FliZ may reflect this ancient relationship rather than being the result of horizontal transfer of gene fragments or convergent evolution.

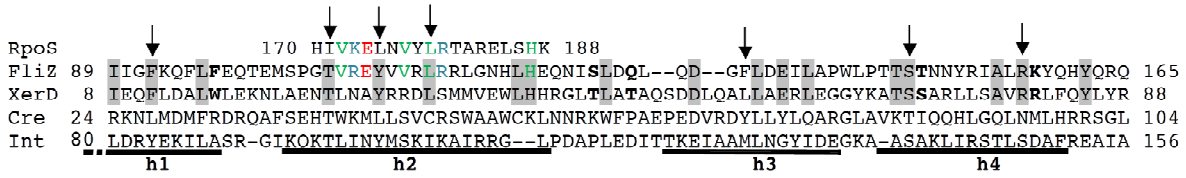

Fig. 5.2: Alignment of the C-terminal part of FliZ with the core-binding domain of three members of the phage integrase family and with the first alpha helix in domain 3 of σ^S (RpoS). Positions that are positively charged in both FliZ and σ^S are coloured in blue, negative charges and other amino acids present in both proteins are printed in red and green, respectively. Identical residues in FliZ and XerD, which shows highest similarity to FliZ, are highlighted by grey background and residues with similar chemical properties present at corresponding positions in both proteins are printed in bold. Arrows indicate strongly conserved residues present throughout the phage integrase family (Swalla et al. 2003), which are also present in FliZ. The alpha helical composition of the core-binding domain of XerD from *E. coli*, Cre from P1 phage and λ phage integrase (Int) is indicated underneath the alignment. The alignment is based on the alignment by Swalla et al. (Swalla et al. 2003) with FliZ added after performance of a conserved domain search using the NCBI CD-search interface (Marchler-Bauer and Bryant 2004).

5.2.3 The role of FliZ in the regulation of motility in *E. coli*

Although several groups have analyzed the role of FliZ in motility in *E. coli* (Mytelka and Chamberlin 1996; Girgis et al. 2007) and *Salmonella* (Ikebe et al. 1999; Kutsukake et al. 1999; Saini et al. 2008), their findings were partly inconsistent and in none of the cases an underlying mechanism has been elucidated in detail (see also 5.2.4). The data presented here demonstrate that in *E. coli* FliZ negatively affects motility through at least two independent

pathways, i.e. by altering the timing of expression of σ^S-dependent EAL genes, and by directly down-regulating *flhDC* transcription.

FliZ affects the expression of several EAL genes. As a direct target of FliZ-regulation, expression of *yciR*, which codes for a functional c-di-GMP-specific phosphodiesterase (Weber et al. 2006), is elevated in the *fliZ* mutant. This elevated expression of *yciR* was here shown to contribute to suppression of the motility defect of the *yhjH* mutant in the absence of FliZ (Fig. 4.34). Together with the findings that ectopic expression of YciR restored motility in the *yhjH* mutant and that this effect required an intact active site EAL motif (Fig. 4.37), this indicates that elevated expression of *yciR* in post-exponentially growing cells can compensate for loss of YhjH through degradation of cyclic di-GMP. Premature expression in the *fliZ* mutant allows YciR to contribute to lowering the levels of c-di-GMP in post-exponentially growing cells and thereby to counteract repression of motility by c-di-GMP bound to YcgR. While this further corroborates how important FliZ is as a timing device in the coordination of motility and curli fimbriae expression, FliZ-mediated regulation of *yciR* expression also represents an example for the concept of temporal sequestration of c-di-GMP synthesizing and degrading enzymes. This aspect will be discussed in more detail below (see 5.3.1.1).

The motility assay with the *yhjH*/*fliZ*/EAL gene triple mutants clearly showed that YjcC and YlaB also contribute to suppression of the non-motile phenotype of the *yhjH* mutant in the absence of FliZ (Fig. 4.34). However, this does not necessarily imply that these enzymes are active phosphodiesterases, able to degrade the c-di-GMP that inhibits motility through binding to YcgR. While enzymatic activity of YciR has been confirmed *in vitro* (Weber et al. 2006), phosphodiesterase activity of YjcC and YlaB has not been demonstrated yet. Thus, until PDE activity of these enzymes has been demonstrated, it remains possible that the contributions of YjcC and YlaB to the observed effects are indirect, e.g. through inhibition of a diguanylate cyclase that affects motility. An example for such an indirect regulation has recently been described in *Salmonella*, where the degenerate EAL protein YdiV, which does not possess PDE activity, activates CsgD expression. YdiV influences CsgD expression by negatively affecting the levels of two PDEs involved in down-regulation of CsgD (Simm et al. 2009). Interestingly, the observation that introduction of a *rtn* mutation into the *yhjH/fliZ* double mutant increased motility, while it did not have any effect when introduced into the *yhjH* single mutant (Fig. 4.34), may also hint at a similar indirect effect mediated by Rtn. Since the influence of Rtn on motility apparently depends on altered expression of another FliZ-dependent factor that affects motility, Rtn-mediated repression of YciR, YjcC or YlaB expression or activity may explain this observation. If Rtn inhibits one of these EAL proteins,

the effect of a *rtn* mutation on motility can only be revealed in the absence of FliZ, because YciR, YjcC and YlaB only affect motility in this background. Future experiments should therefore test whether Rtn affects the expression, levels or activities of one of these EAL proteins. This might reveal interesting new mechanistic and regulatory details concerning the coordination of the multiple GGDEF and EAL proteins present in *E. coli*.

In addition, FliZ also directly interferes with flagellar gene expression by binding to a site directly downstream of the transcriptional start site of the *flhDC* operon. Binding to the *flhDC* upstream regulatory region is consistent with a report published during the course of this work, in which co-immunoprecipitation experiments were used to show that in the gram-negative Enterobacterium and insect pathogen *Xenorhabdus nematophila* FliZ binds to the *flhDC* promoter *in vivo* (Lanois et al. 2008).

The FliZ binding site in the *flhDC* upstream regulatory region, which was determined here, does not seem to be part of a promoter and thus constitutes a conventional operator site that is most likely defined by similarity to the extended -10 element of σ^S-dependent promoters. This shows that FliZ is not necessarily restricted to regulation of σ^S-dependent promoters, which vastly extends the regulatory potential of FliZ. From an evolutionary perspective, inclusion of new genes into the regulon of a protein that is confined to binding to functional promoter sequences will be strongly restricted by stringent sequence constraints due to the requirement for promoter recognition by sigma factors. In contrast, conventional operator sites can more easily be created or altered as a result of minor mutations. For example, a simple base pair exchange creating a sequence with arbitrary similarity to the -10 element of σ^S-dependent promoters could be sufficient to put a new gene under the control of FliZ. Thus, the use of conventional operator sites also greatly extends the evolutionary potential of FliZ.

The repressing effect of FliZ on *flhDC* expression and consequently the entire flagellar gene regulon establishes a negative feedback loop in motility control (Fig. 4.28). This negative feedback control may help to establish an upper limit to flagellar gene expression during post-exponential phase that still allows it to remain responsive to the negative influences that induce down-regulation of flagellar gene expression during entry into stationary phase. A basic requirement for the regulatory system that controls the switch between motility and curli fimbriae-mediated adhesion to work is its sensitivity to changes in the sigma factor competition balance. Sigma factor competition between accumulating σ^S and σ^{70}/σ^{28} seems to contribute to shutting down flagellar gene expression (see 5.1.2). Moreover,

the timing of induction of essential activators of curli fimbriae expression was shown to be highly responsive to alterations in the competitiveness of these sigma factors (see 5.1.3). Thus, over-activation of flagellar gene expression, which would also result in excessive production of σ^{28}, would render the system insensitive to changes in σ^S during the transition into stationary phase. The FliZ-mediated negative feedback loop may therefore act as a homeostatic control system that sets a threshold to σ^{28} expression. Considering that FliZ also globally affects σ^S activity, one of its main tasks may lie in establishing an adequate sigma factor balance that is a prerequisite for the motility to adhesion switch. In this context the finding that FliZ may directly interact with σ^{70} (Fig. 4.26) is particularly interesting, as this interaction may further contributing to this function in a yet to be identified way.

Finally, binding of FliZ to the upstream regulatory region of the *flhDC* regulon may also contribute to the role σ^S plays in the down-regulation of flagellar gene expression. If σ^S and FliZ compete for binding to σ^S-dependent promoters, accumulating σ^S will eventually outcompete FliZ at these promoters. While FliZ may no longer be able to bind to its σ^S-dependent target promoters, it can still bind to operator sites that do not function as promoters, such as the binding site within the *flhDC* upstream regulatory region. Since more FliZ becomes available for binding to these sites when σ^S levels rise, displacement of FliZ from σ^S-dependent promoters may contribute to σ^S-mediated down-regulation of motility during entry into stationary phase.

5.2.4 The role of FliZ in other bacterial species

This study is the first one to elucidate the direct molecular mechanism of FliZ action. However, other groups have reported and analyzed FliZ-mediated effects on motility as well as virulence in different bacterial species. In both *Salmonella* and *X. nematophila*, FliZ regulates the expression of flagellar genes and is involved in the regulation of virulence gene expression. The finding that FliZ binds to the promoter regions of several *X. nematophila* genes *in vivo* is consistent with the data presented here (Lanois et al. 2008). In contrast, a post-transcriptional mechanism was suggested to mediate the effects of FliZ on flagellar gene expression (Saini et al. 2008), on the expression of type 1 fimbrial genes (Saini et al. 2010b) and on SPI1 genes (Lucas and Lee 2001; Kage et al. 2008; Chubiz et al. 2010) in *Salmonella*. However, the molecular mechanism by which FliZ acts has not been clarified in any of these systems, raising the question whether FliZ employs different mechanisms of action in these three species.

An alignment of FliZ proteins from *E. coli*, *S. typhimurium* and *X. nematophila* demonstrates that the potential alpha helix in FliZ, which is involved in DNA binding and mimics the extended -10 element recognition helix of σ^S, is completely conserved in the three proteins (Fig. 5.3A). Furthermore, the FliZ-binding site is 100% conserved in the region downstream of the *E. coli* and *S. typhimurium flhDC* promoters, while sequences further upstream and downstream (in the 5`-untranslated region) show some divergence between these species (Fig. 5.3B). These conservations suggest that the mechanism of FliZ action is the same in these organisms.

Fig. 5.3: Alignments indicating similar mechanisms of action for FliZ in *Escherichia coli*, *Salmonella enterica* and *Xenorhabdus nematophila*. (A) Alignment of FliZ proteins from *Escherichia coli*, *Salmonella enterica* and *Xenorhabdus nematophila*. Identical residues in all three sequences are marked with an asterisk, conserved substitutions are marked with two dots and semi-conserved substitutions with one dot. The putative alpha helix, which closely resembles the first alpha helix in domain 3 of σ^S and is involved in DNA binding by FliZ in *E. coli*, and the corresponding sequences in the other two proteins are marked in red. **(B)** Alignment of the *flhDC* upstream regulatory region of *Escherichia coli* and *Salmonella enterica*. The alignment comprises 266 and 280 nucleotides upstream of the translational start codons of the *E. coli* and *S. enterica flhD* genes, respectively. The FliZ binding site in the *flhDC* upstream regulatory region of *E. coli* that has been determined in this work and the corresponding sequence in *S. enterica* are highlighted in red, the transcriptional start site is coloured in blue, the -10 and -35 elements are printed in grey (the transcriptional start site in *Salmonella* has been reported in (Yanagihara et al. 1999)). The alignments were performed using ClustalW2 (Chenna et al. 2003).

Moreover, it seems unlikely that FliZ employs different mechanisms of action in the regulation of different factors in the same organism, i.e. post-translational regulation of $FlhD_4C_2$ (Saini et al. 2008), but post-transcriptional (Kage et al. 2008) or activity control (Chubiz et al. 2010) of HilD in *Salmonella*. Therefore indirect effects, based on a common DNA-binding mechanism are the simplest and most likely explanation for the different roles FliZ was proposed to assume in the different contexts investigated. FliZ may e.g. inhibit transcription of a factor involved in the proposed post-transcriptional or post-translational control mechanisms. The above finding, that FliZ is able to influence motility through direct transcriptional regulation of the gene encoding the c-di-GMP-specific phosphodiesterase YciR represents a concrete example of such an indirect regulatory link. FliZ-mediated regulation of the central SPI1 regulator HilA was shown to be indirect, through regulation of HilD, a transcriptional activator of *hilA* expression. Since no mechanistic details on the suggested post-transcriptional or post-translational regulation of HilD by FliZ have been elucidated (Kage et al. 2008; Chubiz et al. 2010), participation of a FliZ-dependent factor in HilD regulation may account for this effect. The same applies to the suggested mechanisms for FliZ-mediated regulation of the type 1 fimbrial regulator FimZ and for FliZ-mediated regulation of $FlhD_4C_2$ in *Salmonella*. As also discussed by Saini et al. (2008), FliZ may regulate the expression of a factor directly involved in post-transcriptional control of $FlhD_4C_2$. Interestingly, Saini et al. (2010b) suggested interference of FliZ with promoter activation by FimZ as one possible mechanism for the observed effects on type 1 fimbrial gene expression, which would be consistent with the DNA-binding mechanism identified here. It should be noted, however, that the observation that FliZ might directly interact with σ^{70} (Fig. 4.26) indicates that its mechanism of action may not necessarily be restricted to DNA binding, but may also include effects mediated through protein-protein interaction.

FliZ-mediated activation of SPI1 gene expression was shown to be independent of σ^S (Chubiz et al. 2010). However, Chubiz et al. (2010) also investigated the effect of FliZ on σ^S-dependent gene expression in *Salmonella*. Over-expression of FliZ repressed the expression of two σ^S-dependent genes, indicating that FliZ also acts as a negative regulator of σ^S-dependent gene expression in *Salmonella*. Thus, similar to the situation in *E. coli*, some FliZ-mediated effects seem to be based on repression of σ^S-dependent genes, while other processes affected by FliZ are not controlled by σ^S. Together with the conservation of the putative alpha helix involved in promoter recognition in *E. coli*, this strongly argues that the mechanism through which FliZ acts in *E. coli*, i.e. binding to σ^S-dependent promoters and/or σ^S-promoter-like operator sites by sigma factor mimicry, is conserved in *Salmonella*. Conservation of

sigma factor mimicry as a common mechanism of FliZ action in different species is further supported by a similar phylogenetic distribution of σ^S and FliZ. σ^S is present in γ-, β- and δ-proteobacteria (Chiang and Schellhorn 2010) and FliZ is conserved in the group of Entero- γ-proteobacteria only (Liu and Ochman 2007), therefore restricting the presence of FliZ to species that also posses σ^S.

The major discrepancy between FliZ-mediated regulation in *E. coli* on the one hand and in *Salmonella* and *Xenorhabdus* on the other hand concerns the nature of the effects mediated by FliZ: While it was shown here that FliZ acts as a *repressor* of motility and σ^S-dependent gene expression in *E. coli*, it *activates* flagellar and virulence gene expression in the latter two species (Lanois et al. 2008; Saini et al. 2008).

It cannot be ruled out that FliZ acts as a positive DNA-binding regulator in *Salmonella* and *Xenorhabdus*. However, since the role of FliZ in *Salmonella* and *Xenorhabdus* has only been investigated *in vivo*, the observed differences in the regulatory output of FliZ are more likely due to indirect effects on motility and virulence gene expression. If such an indirect effect is strong enough, it may even override a potential direct inhibition of *flhDC* expression by FliZ, particularly since the above results indicate that, at least in *E. coli*, FliZ only moderately represses *flhDC* expression (Fig. 4.28A). FliZ-mediated repression of a strong inhibitor of motility and/or virulence gene expression would e.g. explain the requirement for FliZ to achieve full motility and virulence in *Salmonella* and *Xenorhabdus*. It is again tempting to speculate that enzymes involved in turnover of c-di-GMP may be involved in this potential indirect regulation by FliZ. Since high c-di-GMP levels are known to generally repress both motility and virulence, the positive role of FliZ in regulation of these two phenotypes in *Salmonella* and *Xenorhabdus* may be based on FliZ-mediated repression of one or several diguanylate cyclases that affect motility and virulence gene expression. *E. coli* and *Salmonella*, although closely related, differ in the number of GGDEF and EAL domain proteins encoded in their genomes (29 in *E. coli* versus 19 in *Salmonella*), with a limited overlap of homologous proteins, and the contributions of single GGDEF and EAL proteins to the regulation of certain phenotypes also differ between these organisms (Hengge 2010b). Thus, differences in FliZ-mediated regulation of motility and virulence between these species may be due to differences in the identity of EAL and GGDEF proteins belonging to the FliZ and/or σ^S regulons in these species. Alternatively, differences in the FliZ dependence of other known or unknown motility and virulence regulators that are not directly involved in c-di-GMP signalling may account for the observed differences.

A good candidate for such a regulator might be the degenerate EAL protein YdiV. YdiV, which does not exhibit any PDE or DGC activity (Simm et al. 2009), was recently shown to inhibit motility by directly interacting with FlhD, thereby inhibiting $FlhD_4C_2$-mediated transcription in *Salmonella* (Wada et al. 2011). Another report suggested that YdiV negatively regulates pathogenicity in *Salmonella*, as a *ydiV* mutation accelerated bacterial killing of macrophages (Hisert et al. 2005). Considering the finding that YdiV regulates $FlhD_4C_2$ activity through direct interaction, YdiV may also directly interact with one of the SPI1 regulators, e.g. HilD. Assuming down-regulation of *ydiV* expression by FliZ, this would be consistent with the proposed FliZ-mediated activity control of HilD in the regulation of SPI1 expression (Chubiz et al. 2010). Thus, potential repression of *ydiV* expression by FliZ in *Salmonella* would firstly explain regulation of SPI1 gene expression by FliZ. It is interesting to note that expression of *ydiV* was shown to be elevated in the absence of *flhDC* expression in *Salmonella* (Wozniak et al. 2009), which may reflect the suggested repression of *ydiV* expression by FliZ. Secondly, differential regulation of *ydiV* expression in *E. coli* and *Salmonella* may then also explain the differences in motility regulation between these two organisms. Differential *ydiV* expression could either be based on differences in FliZ dependence that put *ydiV* under negative FliZ control in *Salmonella* but not in *E. coli*, or result from other regulatory influences establishing differences in *ydiV* expression under the relevant conditions, i.e. low or no expression in *E. coli* versus high expression in *Salmonella.* Consistent with the latter scenario, differences in the expression of *ydiV* between *E. coli* and *Salmonella* have recently been proposed to be responsible for differences in the regulation of flagellar gene expression in response to nutrient conditions (Wada et al. 2011). Expression of *ydiV* is below detection in *E. coli* cells grown in liquid culture (Sommerfeldt et al. 2009). Thus, even if *ydiV* was a FliZ target in both *Salmonella* and *E. coli*, YdiV-mediated regulation may not contribute to the effect FliZ exerts on motility in *E. coli*, since it may not be present under conditions promoting motility. Together, these data suggest YdiV as a potential candidate responsible for differences in FliZ-mediated regulation of motility in *E. coli* and *Salmonella*. The role of this regulator should thus be analyzed in more detail.

In order to identify additional candidates involved in establishing divergent regulation by FliZ, it would also be highly interesting to compare the FliZ regulons of *E. coli*, *S. typhimurium* and *X. nematophila*. Differences in the FliZ regulons between these species may be the result of mutational events that, in the course of evolution, have either led to differences in the composition of the σ^S regulons, thereby also putting different genes under

the control of FliZ, or have created FliZ operator sites that are not part of σ^S-dependent promoters.

Regardless of the mentioned discrepancies in the nature of effects mediated by FliZ, *E. coli* and *Salmonella* show similarities with respect to the physiological function of FliZ.

FliZ-mediated repression of type 1 fimbriae expression and activation of SPI1 gene expression in *Salmonella* establishes a role for FliZ that is reminiscent of the one it assumes in mediating mutual exclusion of motility and curli fimbriae expression in *E. coli* (Saini et al. 2010b). Similar to its function in the lifestyle-transition in *E. coli*, this FliZ-dependent regulation in *Salmonella* is also part of a larger network that was proposed to coordinate factors involved in motility, host cell invasion and colonization/persistence in line with progression through the infection cycle (Saini et al. 2010b). Interestingly, FliZ also has an influence on the timing of gene expression in *Salmonella*, with flagellar and SPI1 genes showing prolonged expression when FliZ was over-produced. In contrast, expression of the negatively FliZ-regulated type 1 fimbrial gene *fimA* was induced slightly earlier in the *fliZ* mutant as compared to the wild-type, similar to the expression of the *E. coli mlrA* and *ydaM* genes described above (Fig. 4.5). An interesting open question is whether this negative regulation by FliZ is based on σ^S dependence of *fimA* expression.

Finally, due to its effect on motility, FliZ also contributes to feedback control of flagellar gene expression in *Salmonella*. However, in this organism FliZ establishes a positive feedback loop, which was suggested to couple class II gene expression to flagellar assembly (Saini et al. 2008; Saini et al. 2010a). Since expression of *fliZ* is regulated by σ^{28}, based on control of the *fliAZY* operon by both class II and III promoters, it is enhanced upon completion of the hook-basal body complex. Thus, the positive effect of FliZ on class II expression was proposed to strongly induce class II expression only upon completion of the first functional hook-basal bodies, in a way similar to the long-established coupling of class III expression to hook-basal body assembly (Saini et al. 2008; Saini et al. 2010a). Interestingly, this implies yet another role of FliZ in regulating the timing of gene expression.

5.3 Regulatory and mechanistic details of curli fimbriae control by the c-di-GMP control module YdaM/YciR illustrate emerging principles of c-di-GMP signalling in *E. coli*

5.3.1 Temporal sequestration essentially determines the regulatory outcome of YdaM and YciR-mediated c-di-GMP regulation

One of the most puzzling and yet mainly unresolved questions in the field of c-di-GMP signalling concerns the mechanisms through which signalling specificity is achieved in cells encoding multiple copies of all the components involved in c-di-GMP signalling. Sequestration of c-di-GMP signalling components was suggested to specifically connect certain c-di-GMP metabolizing enzymes with effector components and target processes (Hengge 2009a).

In the regulation of the asymmetric cell division of *Caulobacter crescentus* cells into a sessile stalked and a motile swarmer daughter cell, several examples for spatial sequestration of DGC and PDE activities can be found. One example is given by the response regulator and DGC PleD, which is targeted to the emerging stalked pole during swarmer-to-stalked cell transition, where it mediates loss of motility, flagellum ejection and stalk biogenesis (Hecht and Newton 1995; Aldridge and Jenal 1999; Aldridge et al. 2003; Paul et al. 2004; Paul et al. 2007). The concomitant sequestration and activation of DGC activity, which is induced by phosphorylation, strongly suggests local c-di-GMP production at the stalked pole. In the plant pathogen *Xanthomonas campestris*, the HD-GYP protein and phosphodiesterase RpfG specifically interacts with two DGCs (Ryan et al. 2010). This interaction was shown to be required for regulation of a subset of RpfG-dependent virulence functions. Since it suggests that these RpfG-GGDEF complexes may specifically affect certain target functions, it may be an example of functional sequestration of c-di-GMP signalling components in micro-compartments. The data presented in this thesis provide several lines of evidence for the concept of temporal sequestration that has been suggested as another mode of specifically connecting certain c-di-GMP signalling components to target functions by restricting their presence and activity to specific time points in the cell cycle or to specific environmental conditions.

5.3.1.1 Temporal sequestration of YciR specifically links it to the regulation of curli fimbriae expression

It was shown here that FliZ coordinates the timing of expression of the c-di-GMP specific phosphodiesterase YciR and that the timing of *yciR* expression determines the functional context in which YciR acts, i.e. which molecular targets and phenotypes are regulated by the activity of this protein. In the regulation of curli fimbriae expression, YciR acts as an antagonist to the diguanylate cyclase YdaM, which synthesizes c-di-GMP essential for transcriptional activation of the curli regulator CsgD (Weber et al. 2006; Pesavento et al. 2008). Whole genome transcription profiling of *yciR* and *ydaM* mutants had revealed that curli fimbriae expression is the only target of YdaM/YciR-mediated regulation during entry into stationary phase (Weber et al. 2006). Moreover, a *yciR* mutation did not affect swimming motility (results by Gisela Becker in (Pesavento et al. 2008)), indicating that when expressed under wild-type conditions, YciR is functionally restricted to regulate curli fimbriae expression during entry into stationary phase, when *yciR* expression is highest.

Premature expression of YciR in post-exponential phase, as observed here in the *fliZ* mutant and with ectopic expression of YciR, exposes YciR to a different set of c-di-GMP signalling components and targets. Presence in post-exponential phase allows YciR to degrade the motility-inhibiting c-di-GMP, i.e. rewires it to a new target processes. Thus, FliZ-mediated regulation of *yciR* expression represents an excellent example for the concept of temporal sequestration. Since elevated post-exponential expression of other EAL genes (*yjcC*, *ylaB*), which do not influence motility under wild-type conditions, contributes to restoring motility in the *yhjH/fliZ* mutant, YciR does not seem to be the only PDE whose target spectrum is determined by tight control of expression.

The notion that specific expression patterns connect distinct DGCs and PDEs to specific effector and target systems is further supported by reports suggesting that differences in expression patterns may cause functional divergence between the components of the c-di-GMP signalling system in different strains of the same species. For example, higher expression levels of the GGDEF protein YedQ, which have been observed in certain *E. coli* strains, seem to endow the cell with a way to bypass CsgD/AdrA-dependent induction of cellulose synthesis (Da Re and Ghigo 2006). Thus, simple variations in the expression patterns of the corresponding genes, e.g. due to promoter mutations that alter the expression pattern of a GGDEF or EAL gene (e.g. through altering its FliZ dependence) may also account for some of the divergence in function between homologous c-di-GMP signalling proteins of closely related species such as *Salmonella* and *E. coli*.

The finding that suppression of the non-motile phenotype of the *yhjH* mutant by mutation of *fliZ* could only be observed at 28°C, but not at 37°C (Fig. 5.32) may indicate that YciR is subject to temperature control, which may further restrict its activity and ability to affect target processes to specific conditions. Since ectopically expressed YciR also restores motility in a *yhjH* mutant at 37°C (data not shown), it is unlikely that its activity is regulated by temperature. However, expression of *yciR* is also similar at 28°C and 37°C (Weber et al. 2006; Sommerfeldt et al. 2009). Thus, temperature regulation of YciR may act at the protein level, but may require an additional factor that is titrated when YciR is present at unusually high levels during ectopic expression. Alternatively, YjcC and YlaB, which also contribute to suppression of the motility defect in the *yhjH/fliZ* mutant, may be repressed in their activities at 37°C.

5.3.1.2 H-NS establishes a YdaM/YciR balance that allows for induction of curli fimbriae expression upon entry into stationary phase

In addition to FliZ, the nucleoid-associated protein H-NS was also found to control the expression of *yciR* and *ydaM* (Fig. 4.40). During entry into stationary phase H-NS activates *ydaM* expression and represses *yciR* expression, which can be expected to shift the YdaM:YciR stoichiometry, thus explaining the activating effect H-NS has on *csgD* expression. This illustrates that transcriptional regulation contributes to determining the target spectrum of certain DGCs and PDEs, not only by establishing the concomitant presence of distinct DGCs, PDEs and their effector and target components, but also by establishing a balance between antagonizing activities within a c-di-GMP control module. Another example for this kind of regulation is the concomitant down-regulation of *yhjH* expression and induction of *yegE/yedQ* expression during entry into stationary phase, which is a prerequisite for allowing YegE/YedQ-synthesized c-di-GMP to contribute to down-regulation of motility and induction of *csgD* expression (see above).

The role of H-NS-mediated regulation in temporal sequestration of the YdaM/YciR control module is further emphasized by the observation that *yciR* expression is elevated in the *hns* mutant throughout the entire growth cycle, i.e. also during post-exponential phase. Thus H-NS, like FliZ, seems to contribute to insulating YciR from c-di-GMP-mediated motility regulation.

Interestingly, a mutation in *ycgR*, which encodes the c-di-GMP effector in motility control, and over-expression of YhjH were found to suppress the motility-defect of a *hns* mutant that ectopically expresses *flhDC* (i.e. the motility defect was not due to reduced *flhDC*

expression in the *hns* mutant). Thus, the motility-defect of this *hns* mutant seems to be due to elevated c-di-GMP levels that interfere with flagellar function, indicating that H-NS is either required for YhjH expression or represses the expression of other DGCs that are able to affect motility. H-NS is known to strongly inhibit σ^S synthesis and promote σ^S turnover during exponential growth; a *hns* mutant thus shows higher exponential phase expression of many σ^S-dependent genes (Barth et al. 1995; Yamashino et al. 1995), which probably also explains the higher exponential phase expression of *ydaM* in the *hns* mutant (Fig. 4.40). Therefore expression of the σ^S-dependent GGDEF proteins YegE and YedQ, which contribute to down-regulation of motility during transition into stationary phase, may also be higher during exponential phase in the *hns* mutant, and c-di-GMP synthesis by these enzymes may be responsible for the motility defect in the absence of *hns*. Together these data suggest that H-NS plays an important and complex role in coordinating the expression of DGCs and PDEs in *E. coli*.

In curli control, the role of H-NS is not restricted to the regulation of *ydaM* and *yciR* expression. In addition to the mentioned effect on σ^S, which can be expected to result in a strong repression of curli fimbriae expression during exponential phase due to the multiple input that σ^S has into the curli control cascade, H-NS also seems to directly repress expression of the curli structural operon *csgBAC* (Olsen et al. 1993; Arnqvist et al. 1994). H-NS-mediated repression of curli fimbriae expression is relieved upon entry into stationary phase, when the repressing influence of H-NS on σ^S is lost and H-NS may shift the YdaM:YciR stoichiometry towards YdaM, thereby inducing *csgD* and in turn *csgBAC* expression. Considering these manifold effects H-NS exerts on both motility and curli gene expression, it is therefore likely to assume a central role in the regulation of the switch from the motile to the adhesive lifestyle.

5.3.2 YciR is a multifunctional protein with a regulatory potential that is not restricted to its phosphodiesterase activity

An intact EAL motif was here shown to be required for the positive effect post-exponentially expressed YciR exerts on motility, but not for repression of curli fimbriae expression during entry into stationary phase. Thus, YciR seems to influence motility through degradation of c-di-GMP, while its role in antagonizing the diguanylate cyclase YdaM in the induction of *csgD* transcription does not seem to be based on simple degradation of the c-di-GMP synthesized by YdaM.

In addition to mediating synthesis and degradation of c-di-GMP, an increasing number of GGDEF and EAL domains have been demonstrated to assume functions that do not imply these enzymatic activities. Some of these functions are completely independent of c-di-GMP signalling, as seen e.g. with the antagonistic activity that the EAL protein YcgF exerts against the DNA-binding repressor YcgE (Tschowri et al. 2008). Other degenerate GGDEF and EAL domains still function in c-di-GMP signalling but have adopted new roles. Catalytically inactive GGDEF and EAL domains that retained their ability to bind c-di-GMP at conserved I-sites or degenerate active sites, respectively, can e.g. function as c-di-GMP-binding effector proteins (Beyhan et al. 2008; Duerig et al. 2009; Navarro et al. 2009; Newell et al. 2009; Qi et al. 2010) and GTP binding to a degenerate GGDEF domain is involved in allosteric regulation of the enzymatic activity of a neighbouring EAL domain (Christen et al. 2005).

Thus, YciR is not the first GGDEF/EAL protein that works through a mechanism, which does not imply an enzymatic activity against c-di-GMP. It is, however, the first example of a GGDEF/EAL protein that influences c-di-GMP signalling through two different mechanisms, one of which involves degradation of c-di-GMP, while the other, yet uncharacterized mechanism does not require its PDE activity. The latter mechanism plays a role in the regulation of curli fimbriae expression, where YciR functions as an antagonist to YdaM. It therefore seems possible that the PDE activity-independent effect of YciR is based on direct interaction with YdaM. Since GGDEF domains are known to require dimerization for enzymatic activity, one could e.g. envisage the formation of catalytically inactive YdaM-YciR heterodimers in which the proteins interact through their GGDEF domains. Such an engagement of YciR and YdaM in a confined microcompartment may also prevent YciR from affecting other c-di-GMP-dependent processes through its PDE activity and thereby contribute to establishing signalling specificity of YciR: While YciR is potentially able to influence other c-di-GMP-dependent processes through its PDE activity, a direct and specific interaction between YciR and YdaM, may force it to “concentrate” on inhibition of YdaM as soon as the latter accumulates in the cell. The PDE-independent function of YciR is a first indication for a direct interaction between the components mediating c-di-GMP control of curli fimbriae expression that eventually needs to be demonstrated by biochemical studies, which are currently performed in the Hengge group (Sandra Lindenberg and Regine Hengge, unpublished results).

5.3.3 Other diguanylate cyclases may contribute to c-di-GMP-dependent induction of curli fimbriae expression

The observation that a *ydaM/yciR* double mutant still shows elevated curli fimbriae expression indicated the presence of c-di-GMP generated by other DGCs, which are also subject to inhibition by YciR (Fig. 4.38). An alternative explanation is, that in addition to antagonizing YdaM, YciR may exert a c-di-GMP-independent inhibitory effect on MlrA, e.g. through direct binding to the transcription factor. In this case, c-di-GMP-independent induction of *csgD* transcription by MlrA may be possible in the absence of YciR. In order to test the first hypothesis, possible contributions of several GGDEF genes to curli fimbriae induction in the *yciR* mutant were analyzed, but mutations in none of the GGDEF genes tested reduced *csgB* expression in this mutant (Fig. 4.39). It is possible that, in addition to YdaM, which seems to play the largest role in curli fimbriae induction, several other DGCs collectively contribute to activating curli fimbriae expression in the *yciR* mutant. If, however, curli fimbriae expression is maximally induced in the *yciR* mutant, smaller contributions of single DGCs may not be detectable in this background. The *ydaM/yciR* double mutant, which does not show maximal induction of curli fimbriae expression, may therefore represent a more appropriate background for introducing additional GGDEF gene mutations.

In addition to clarifying the identity of other DGCs possibly contributing to curli fimbriae expression, it will also be interesting to determine whether the potential YciR-mediated inhibition of these enzymes requires its PDE activity. The fact that the motility assays confirmed that YciR has PDE activity *in vivo* raises the question whether this PDE activity plays any role in c-di-GMP control of curli fimbriae expression and how it is coordinated with the PDE-independent function of YciR.

All data presented here are consistent with a hypothetical model of YdaM/YciR-mediated curli regulation that assumes a bifunctional role of YciR: While YciR may inhibit YdaM through protein-protein interaction, its PDE activity may play a role in inhibiting other DGCs that also synthesize c-di-GMP for induction of curli fimbriae expression. As discussed above, interaction between YdaM and YciR may not only inhibit diguanylate cyclase activity of YdaM, but it may also interfere with the PDE activity of YciR. This is consistent with all observations made in the *ydaM* and *yciR* single and double mutants. It would e.g. explain why expression is extremely low in the *ydaM* mutant, even though the situation in the *ydaM/yciR* double mutant suggests that other DGCs contribute to activation of curli fimbriae expression. According to this model, PDE activity of YciR is not inhibited in the absence of YdaM,

therefore YciR should degrade all curli-relevant c-di-GMP synthesized by other DGCs, which results in a shut-down of curli fimbriae synthesis in the *ydaM* mutant. Consistent with this, expression of YdaM-EE334/335AA, which is defective in c-di-GMP synthesis, still weakly restores curli synthesis in the *ydaM* mutant (Fig. 5.36A) (note that this effect can be expected to be even stronger in a background that does not show arabinose-induced repression of curli fimbriae expression). This indicates that YdaM indeed has a DGC activity-independent effect on curli expression. Inhibition of YciR-mediated degradation of c-di-GMP synthesized by other DGCs may thus explain the residual curli induction observed in the presence of this YdaM mutant.

The observation that the EAL-motif mutant YciR-E440A is still able to completely repress curli fimbriae synthesis (Fig. 4.36B), although, according to this model, it should only inhibit YdaM but allow for accumulation of c-di-GMP synthesized by the other DGCs, may be an artefact of over-expression of YciR. When present in excessive amounts, YciR may interact non-specifically with other DGCs.

As mentioned above, a c-di-GMP-independent inhibitory effect of YciR against MlrA represents an alternative to this model, which is also consistent with the data presented here.

Together the data presented on c-di-GMP control of curli fimbriae synthesis indicate the presence of a multitude of mechanisms ensuring accumulation of the second messenger during entry into stationary phase. By providing evidence for several modes of sequestration they further corroborate that signalling specificity of a specific protein involved in c-di-GMP signalling is not necessarily an intrinsic property of the protein, but rather depends on factors determining the concomitant presence and balanced activity of other c-di-GMP signalling components.

5.3.4 MlrA binds to a σ^S-dependent promoter-like region upstream of the actual *csgD* promoter

While the results discussed above provide further details on the mechanisms of action of the GGDEF and EAL proteins involved in c-di-GMP-mediated curli fimbriae regulation, the identity of the effector that binds the second-messenger and mediates changes in *csgD* transcription remains unknown. The MerR-like transcriptional regulator MlrA is a potential candidate for this c-di-GMP effector, since it is required for activation of *csgD* transcription but, similar to YdaM and YciR, does not seem to regulate any other genes during entry into

stationary phase, under conditions promoting curli fimbriae formation (Weber et al. 2006; Weber 2007).

Here the DNA-binding site of MlrA within the *csgD* promoter was determined. MlrA binds to a site upstream of the known *csgD* promoter. An almost identical binding site (extending 6 nucleotides further upstream than the one identified here) has recently been identified in another *E. coli* K12 strain (Ogasawara et al. 2010b). The MlrA-binding site does not overlap with the known binding sites of other positive regulators of *csgD* transcription, but binding of the negative regulator CpxR, as well as H-NS extends into the MlrA-binding region (Ogasawara et al. 2010a; Ogasawara et al. 2010b). The exact mechanism through which MlrA induces *csgD* transcription from this site, which is situated more than 100 nucleotides upstream of the transcriptional start site, is not yet known. MlrA belongs to the family of MerR-like transcriptional regulators that activate transcription of target genes through the introduction of conformational changes in their operator sites, which results in an optimal positioning of the -10 and -35 elements for recognition by RNAP (Brown et al. 2003). Thus, MlrA binding may promote conformational changes in the upstream regulatory region of the *csgD* gene that could e.g. affect binding and activity of some of the many other regulators binding to this region, as has also been suggested by Ogasawara et al. (2010). Most of the MerR-like regulators activate transcription upon binding of small ligands and this binding is thought to change the conformation of the DNA-bound regulator from a repressing to an activating state. Thus, it is tempting to speculate that c-di-GMP binding triggers this change in MlrA.

Interestingly, the identified MlrA-binding site overlaps with a sequence that almost perfectly matches the consensus sequence of σ^S-dependent promoters. Within this putative σ^S-dependent promoter, MlrA binds to the -35 element and to parts of the spacer region which is consistent with the typical binding of MerR-like regulators to sites within the spacer region of target promoters (Brown et al. 2003). Although this suggested that this sequence may represent an additional MlrA- and σ^S-dependent *csgD* promoter, no transcriptional activity starting from this sequence had been detected under the conditions under which curli fimbriae expression has also been analyzed here, i.e. during entry into stationary phase in LB medium at 28°C (Weber 2007). Thus, while the role of this σ^S-dependent promoter-like sequence remains elusive, the MlrA binding site identified here indicates that the MerR-like regulator MlrA obviously employs an unorthodox mechanism to affect *csgD* expression from a binding site upstream of the actual promoter. Details of this mechanism need to be analyzed in future experiments.

6. References

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