Functional characterization of the transcription factor YjjQ in *Escherichia coli*

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Katrin Dreck

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Berichterstatter: Prof. Dr. Karin Schnetz

Prof. Dr. Jürgen Dohmen

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Zusammenfassung

YjjQ ist ein Transkriptionsfaktor des FixJ/NarL-Typs, der in kommensalen und pathogenen *E. coli* wie enterohämorrhagischen *Escherichia coli* (EHEC), uropathogenen *E. coli* (UPEC) sowie in *Citrobacter* und *Enterobacter* konserviert ist. YjjQ wurde zuvor mit bakterieller Virulenz, Motilität und Adhäsion in Zusammenhang gebracht. Eine Microarray Analyse durchgeführt in *E. coli* K-12 und dem UPEC Stamm CFT073 zeigte, dass *yjjQ*-Überexpression in einer signifikanten Repression von mehreren Loci resultiert.

Einer der durch *yjjQ*-Überexpression wesentlich reprimierten Loci ist der *bcs*-Locus verantwortlich für die Synthese von Cellulose. Dieses Exopolysaccharid ist eine Hauptkomponente der bakteriellen Extrazellulären Matrix (ECM). Weitere Bestandteile der ECM sind adhäsive amyloide Fasern genannt Curli. Die ECM erleichtert Zell-Zell- und Zell-Oberfläche-Kontakte, wodurch sie erheblich zur Bildung von Biofilmen beiträgt. Diese Oberflächen-assoziierten multizellulären Gemeinschaften repräsentieren den vorherrschenden Lebensstil von Bakterien. Wildtyp Enterobacteriaceae, die Cellulose und Curli co-exprimieren, weisen eine markante Kolonienmorphologie auf Congo Rot Indikatorplatten auf welche als “red, dry and rough” (rdar) Morphotyp bezeichnet wird.

Eine vermeintliche Repression des *bcs*-Locus durch YjjQ legt nahe, dass dieser Transkriptionsfaktor die Zusammensetzung der ECM beeinträchtigt. Aus diesem Grund wurde die Auswirkung der Expression von YjjQ auf den Morphotyp von Wildtyp und mutierten UPEC 536 Stämmen untersucht. YjjQ schwächt den rdar Morphotype deutlich ab. Um den Einfluss von YjjQ auf die Biofilm-Bildung von *E. coli* K-12 und UPEC 536 Stämmen zu analysieren wurde ein Biofilm Assay durchgeführt. Bakterielle Adhäsion an Polystyrol wird wesentlich durch YjjQ reprimiert. Diese Daten implizieren, dass YjjQ als ein Suppressor von multizellulärem Verhalten und möglicherweise Adhäsion in Enterobacteriaceae fungiert.

Ein weiterer mutmaßlicher durch YjjQ negativ regulierter Locus ist *flhDC*, welcher den Hauptregulator der flagellären Motilität kodiert. Dies weist darauf hin, dass YjjQ bakterielle Motilität negativ kontrolliert. Aus diesem Grund wurde die Wirkung von YjjQ auf die Motilität von *E. coli* K-12 und UPEC 536 Stämmen untersucht. Dies zeigte, dass Motilität erheblich durch YjjQ reprimiert wird. Dies weist darauf hin, dass dieses Protein neben der Inhibition der Biofilm-

Bildung auch ein Suppressor der flagellären Motilität ist. Diese neu identifizierten Funktionen von YjjQ legen nahen, dass dieser Transkriptionsfaktor ein wichtiger Regulator ist, der in das Netzwerk, welches Adhäsion versus Motilität kontrolliert, involviert ist.

Abstract

YjjQ is a FixJ/NarL-type transcriptional regulator conserved in commensal and various pathogenic bacteria such as enterohaemorrhagic *Escherichia coli* (EHEC), uropathogenic *E. coli* (UPEC) as well as *Citrobacter* and *Enterobacter*. YjjQ was previously implicated to play a role in bacterial virulence, motility, and adhesion. Microarray analysis performed in *E. coli* K-12 and UPEC strain CFT073 revealed that *yjjQ* overexpression results in significant downregulation of multiple loci.

One of the putative loci considerably repressed by YjjQ is the *bcs* locus dedicated to cellulose synthesis. This exopolysaccharide is a major component of the bacterial extracellular matrix (ECM). Additional ECM constituents are adhesive amyloid fibers called curli. The ECM facilitates cell-cell and cell-surface contacts thereby largely contributing to biofilm formation. These surface-associated multicellular communities represent the prevalent bacterial lifestyle. Wild-type Enterobacteriaceae co-expressing cellulose and curli exhibit a distinctive colony morphology on Congo Red indicator plates called the red, dry and rough (rdar) morphotype.

Putative repression of the *bcs* locus by YjjQ suggests that this transcription factor may affect ECM composition. Therefore the effect of YjjQ expression on the morphotype of wild-type and mutant UPEC 536 strains was investigated. YjjQ was found to considerably weaken the rdar morphotype compared to the control. To examine the influence of YjjQ on biofilm formation of wild-type and mutant *E. coli* K-12 and UPEC 536 strains a biofilm assay was performed. YjjQ substantially represses bacterial adhesion to polystyrene. These data suggest that YjjQ acts as a suppressor of multicellular behavior and possibly adhesion of Enterobacteriaceae.

Another putative target locus negatively regulated by YjjQ is *flhDC* encoding the master regulator of flagellar motility. This implies that YjjQ may impinge upon bacterial motility. Therefore the impact of YjjQ on motility of wild-type and mutant *E. coli* K-12 and UPEC 536 strains was investigated. YjjQ was found to substantially repress motility suggesting that besides inhibiting biofilm formation, it also acts as a suppressor of flagellar motility. These newly identified functions of YjjQ indicate that this transcription factor may be an important regulator involved in the network controlling adhesion versus motility.

1. Introduction

Escherichia coli is one of the most commonly used model organisms in molecular biology and a valuable tool in biotechnology. Naturally occurring *E. coli* may encounter many detrimental conditions such as desiccation, starvation or acid stress. Therefore bacteria have to be prepared to react appropriately to these threads by mounting immediate responses. One universal survival strategy of bacteria confronted with stress is the formation of biofilms. These adhesive multicellular consortia offer plenty of benefits. Bacteria enclosed in a biofilm are protected against virtually all kinds of aggressions from the outside world. The biofilm lifestyle can be attributed to pronounced gene regulation governed in large part by transcription factors. In this way these regulatory proteins account for the broad success of bacteria in withstanding external assaults. In this thesis, the role of the FixJ/NarL-type transcription factor YjjQ is investigated in *E. coli* with a focus on biofilm formation.

1.1 *Escherichia coli*

The family of Enterobacteriaceae is a large group of gram-negative bacteria consisting of multiple species such as *Salmonella* spp., *Klebsiella* ssp., *Yersinia* spp., and *Escherichia* spp. (Sanderson, 1976). *Escherichia coli* co-exists as a symbiotic organism in vertebrates, being most prevalent in mammals including humans. It inhabits the lower gastrointestinal tract thereby constituting the normal gut flora of warm-blooded animals (Hartl & Dykhuizen, 1984). The non-pathogenic *E. coli* strain K-12 was isolated in 1922 from a stool sample of a patient convalescent from diphtheria and is since then used routinely in microbiological and genetic research (Bachmann, 1972).

The gastrointestinal tract, due to its trait of being a comfortable ecological niche for microbes, is prone to be infected with a variety of well-adapted pathogenic bacteria. Among the intestinal pathogenic *E. coli* found in humans are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC), all of them causing severe diarrhea (Kaper *et al.*, 2004). However, extraintestinal pathogenic *E. coli* (ExPEC) that colonizes tissues and organs other than the gut can elicit serious diseases as well. ExPEC include neonatal meningitis-causing

E. coli (NMEC), uropathogenic *E. coli* (UPEC), and avian pathogenic *E. coli* (APEC) (Smith *et al.*, 2007). APEC evokes systemic infections in domesticated birds causing high mortality in poultry such as chickens and turkeys (Mokady *et al.*, 2005). APEC strains are very similar to the reference UPEC strains as evident by comparison of their genome (Johnson *et al.*, 2007, Moulin-Schouleur *et al.*, 2007).

UPEC is the primary cause of community-acquired and nosocomial urinary tract infections (UTIs), one the most common bacterial infections in humans at all. Especially women suffer from UTIs and will probably experience more than one infection in their lifetime because UTIs have a high rate of recurrence (Bower *et al.*, 2005). UPEC employs a diverse repertoire of virulence factors, as it harbors more genes encoding iron acquisition systems (e.g. siderophores), adhesins (e.g. type 1 fimbriae), and secreted toxins (e.g. α-hemolysin, cytotoxic necrotizing factor 1, and autotransporters) than *E. coli* K-12 laboratory strains and commensal isolates (Wiles *et al.*, 2008). These virulence-associated genes are usually organized in pathogenicity islands that have been acquired by horizontal gene transfer (Hacker & Kaper, 2000, Gal-Mor & Finlay, 2006).

UPEC has evolved a number of strategies to evade the defense system of the host (Hunstad & Justice, 2010). In the course of an UTI, UPEC invades urothelial host cells and establishes biofilm-like intracellular bacterial communities (IBCs) (Anderson *et al.*, 2004). UPEC preferentially colonizes the bladder causing uncomplicated cystitis, but it can also ascend through the ureters into the kidneys, causing acute pyelonephritis. Severe cases of pyelonephritis can lead to renal failure or the entrance of UPEC into the bloodstream thereby causing systemic infections such as sepsis (Sivick & Mobley, 2010).

Two sequenced UPEC strains frequently used in molecular research are the human pyelonephritis isolates CFT073 (O6:K2:H1) and 536 (O6:K15:H31) (Welch *et al.*, 2002, Brzuszkiewicz *et al.*, 2006). A conventional mode of bacterial classification is based on different cell surface structures. By this method pathogenic *E. coli* are categorized into so-called serotypes. The O groups characterize the lipopolysaccharide (LPS) moiety of the cell wall. The K groups characterize capsular polysaccharides. The H groups characterize proteinaceous components belonging to flagella (Orskov *et al.*, 1977). These surfaces structures elicit immune

responses in the host and therefore represent antigenic determinants. Furthermore, they also largely contribute to biofilm formation.

1.2 Biofilm formation

The ability to adapt quickly to various surroundings is essential for bacteria as they are exposed to constantly changing environmental conditions. This adaptation involves modification of signal transduction mechanisms that communicate information from the outside into the cell. Most of these mechanisms ultimately result in alteration of transcription in order to achieve the most favorable lifestyle under the given circumstances. The two main bacterial lifestyles are the planktonic lifestyle and the biofilm lifestyle. Planktonic bacteria are freely swimming and therefore highly motile. But the planktonic lifestyle occurs on the single cell level which represents a rather unfavorable bacterial existence. In contrast to the planktonic lifestyle which is rarely found in nature, the majority of bacteria has a strong tendency towards a surface-associated mode of growth (Zobell, 1943). On that account biofilms represent the prevalent bacterial lifestyle in most ecosystems.

Biofilms are characterized by the formation of cellular communities with coordinated behavior that thrive on different surfaces and interfaces. This process depends on the production of a cohesive conglomeration of versatile biopolymers by the bacteria themselves. This vast array of exopolysaccharides, secreted proteins, and cell-surface adhesins is collectively termed the extracellular matrix (ECM; also termed EPS for extracellular polymeric substance) (Branda *et al.*, 2005, Flemming & Wingender, 2010).

Aggregation of bacterial cells belonging to different species facilitates acquisition of new genetic traits, which enhances the genetic diversity of these bacteria (Davey & O'Toole, 2000). Such multi-species biofilms prevail in natural settings, whereas single-species biofilms play a fundamental role in a variety of infections (e.g. *Pseudomonas aeruginosa* in cystic fibrosis) (see chapter 5.3). Biofilms are the cause of numerous persistent and chronic infections, as they often are resistant to antimicrobial agents (Mah & O'Toole, 2001). Subinhibitory concentrations of antibiotics can actually lead to biofilm formation in the first place (Hoffman *et al.*, 2005).

Reduced susceptibility of biofilm bacteria to drugs is a crucial problem for treatment of these infections (Ito *et al.*, 2009).

Biofilm formation is first of all dependent on the specific attributes of each particular bacterial strain as well as on the properties of the surface being colonized. Beyond that, manifold environmental cues such as temperature, osmolarity, pH, and nutrient, iron as well as oxygen availability exert a considerable influence on biofilm formation (O'Toole *et al.*, 2000). Further signals stimulating sessility can also be produced and secreted by the bacteria themselves. These so-called autoinducers accumulate extracellularly thereby correlating with population density. At high concentrations, autoinducers trigger signal transduction cascades that lead to biofilm formation. This mechanism of cell-cell communication in bacteria is called quorum sensing (López *et al.*, 2010).

Structural organization and the formation of differentiated microcolonies are hallmarks of biofilms. A biofilm forms as a sequence of defined stages beginning with the reversible attachment of bacterial cells to a surface. In the next stage the ECM is produced resulting in committed attachment. After that the early biofilm shape develops followed by the maturation into an elaborated architecture. The final step is the release of individual cells or whole groups of cells from the biofilm (Stoodley *et al.*, 2002). Under laboratory conditions, the characteristic biofilm architecture can be observed on special indicator plates as rugose or wrinkled colony morphology. Bacteria expressing biofilm determinants display a peculiar phenotype called the red, dry, and rough morphotype (Römling, 2005) (see chapter 2.3).

The decision for a sedentary lifestyle is a complex course of events that involves all levels of gene regulation. Synthesis and assembly of adhesive structures are costly and have to be coordinated with the expression of motility structures. For instance, the transition from motility to adhesion requires down-regulation of flagellar motility at the transcriptional, post-transcriptional, protein activity and protein degradation levels. Consequently, adhesion and motility can be considered as mutually exclusive cellular states. This is reflected in a distinctive inverse regulation of the pathways leading to the development of either adhesion or motility (Pesavento *et al.*, 2008). Extrinsic stimuli detected by specialized cell surface receptors are transmitted via signaling cascades to transcriptional regulators. These mediators consequently

target the adequate genes to be either activated or silenced under the conditions perceived. If transition to a community-based lifestyle is triggered, expression of motility-associated genes is downregulated while expression of genes encoding adhesion structures is upregulated.

The model described above represents a simplistic version of biofilm formation because adhesion and the biofilm lifestyle are not synonymous terms. It was shown that motility is an important process especially during the early stages of biofilm formation (Pratt & Kolter, 1998). However, this correlation is strongly dependent on the growth conditions of the bacteria. Furthermore, synthesis and activity of flagella influences the elaborated architecture of mature biofilms in *E. coli* (Wood *et al.*, 2006). Besides transcription factors, the bacterial second messenger 3',5'-cyclic dimeric guanosine monophosphate (c-di-GMP) embodies a pivotal regulator of the transition between bacterial lifestyles. Moreover, small regulatory RNAs (sRNAs) contribute largely to modulating the output of signal transduction pathways leading to biofilm formation. The involvement of c-di-GMP and sRNAs in the regulation of ECM production is described in the chapters 1.3.3 and 1.3.4, respectively.

1.3 Regulation of extracellular matrix production in *Escherichia coli*

The extracellular matrix (ECM) is a complex structure of different constituents that enables bacteria to interact adequately with their environment. The ECM alone can account for over 90% of the dry mass of a biofilm and therefore plays an important role for its structural integrity. In Enterobacteriaceae, a large part of the ECM is made up of protein appendages called curli and the polysaccharide cellulose, but water is by far the prevalent component. The highly hydrated matrix embeds bacterial cells in a tightly packed network which confers mechanical and chemical protection and enhances resistance to environmental stresses. In addition to providing a survival advantage in the face of hostile conditions, the ECM also facilitates cell-cell and cell-surface contacts (Branda et al., 2005, Flemming & Wingender, 2010).

1.3.1 Curli protein fibers

Curli are the major proteinaceous constituents of the bacterial ECM (Larsen *et al.*, 2007). These amyloid fibers consist of extremely stable β sheet-rich polymers with adhesive properties that promote cell aggregation and community behavior, both processes being prominent features of biofilms. But curli not only provoke interactions between individual bacteria, they also support attachment to various biotic and abiotic surfaces which also contributes largely to biofilm formation (Blanco *et al.*, 2012, Prigent-Combaret *et al.*, 2000). Moreover, curli have been implicated to play roles in host colonization, invasion, and pathogenesis (Barnhart & Chapman, 2006). Curli fibers of *E. coli* are classified as functional amyloids because they serve at least one specific purpose for the organism (Epstein & Chapman, 2008, Wang & Chapman, 2008). But amyloid structures can also be found within deposits of misfolded proteins causing harm in human tissues and organs like the brain. For that reason amyloids are associated with neurodegenerative diseases such as Alzheimer's and Parkinson's, so-called amyloidoses (Chiti & Dobson, 2006).

In Enterobacteriaceae there are two homologous but inconsistently designated operons dedicated to curli expression. Nevertheless the protein products of the *csg* (curlin subunit gene) operons in *E. coli* and the *agf* (thin aggregative fimbriae) operons in *S. typhimurium* exert analogous functions (Römling *et al.*, 1998). The *csgDEFG* operon encodes proteins required for curli assembly (Hammar *et al.*, 1995) (Figure 1). The FixJ/NarL-type transcription factor CsgD in turn activates transcription of the *csgBAC* operon wherein *csgA* codes for the predominant structural component of extracellularly polymerized curli (Chapman *et al.*, 2002). The *csgBAC* operon is repressed by the global repressor H-NS (Arnqvist *et al.*, 1994). CsgD plays a critical role in ECM production because besides inducing curli gene expression, it indirectly modulates cellulose synthesis by activating the protein YaiC responsible for c-di-GMP synthesis (Römling *et al.*, 2000) (see chapter 1.3.3). CsgD therefore represents a master regulator of ECM production. In addition, CsgD directly represses transcription of operons involved in flagella synthesis by binding to a site in the regulatory region that overlaps with the binding site for the master regulator of flagellar motility (Ogasawara *et al.*, 2011). This is just one example for the exceptionally complex reciprocal regulation of motility and adhesion.

The regulation of curli expression is responsive to different environmental conditions such as temperature and salt concentration of the growth medium. In general, *E. coli* laboratory strains like K-12 derivatives have poor adhesive capacities because of low curli expression levels. Curli are maximally expressed at temperatures below 37°C in media without salt (Olsén *et al.*, 1989, Bokranz *et al.*, 2005). But when *E. coli* is grown as a biofilm, it is also able to produce curli at 37°C which enhances adherence to epithelial host cells (Kikuchi *et al.*, 2005). It is speculated that curli expression below 37°C is an adaptation mechanism of bacteria to survival outside an animal host (Prigent-Combaret et al., 2000, Olsén *et al.*, 1993).

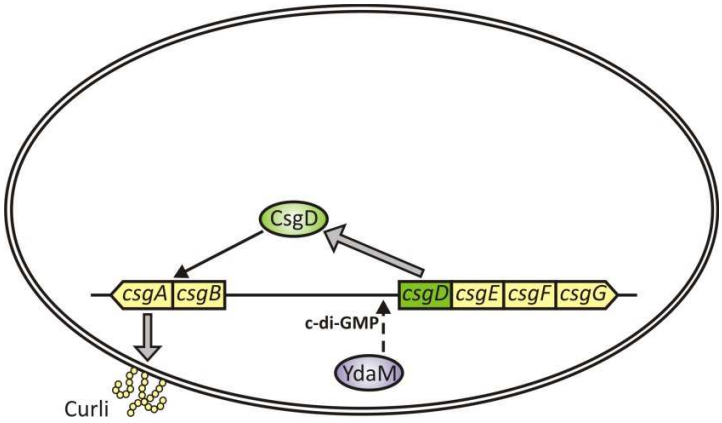


Figure 1: Regulation of *csg* gene expression in *E. coli* leading to curli synthesis.
Transcription of the *csgDEFG* operon is stimulated by the second messenger c-di-GMP produced by YdaM. Expression of the FixJ/NarL-type transcription factor CsgD induces transcription of the *csgBA* operon. Export and subsequent polymerization of CsgA and CsgB leads to extracellular assembly of curli.

The *csgD* promoter is the central part of one of the most complex signaling networks found in the *E. coli* genome. This intricate regulation involves an extensive interplay of positive as well as negative regulators (Ogasawara *et al.*, 2010). The *csgD* promoter is directly bound or indirectly affected by at least 10 different transcriptional regulators such as the general stress sigma factor RpoS, the global repressor H-NS, and the global transcriptional regulator Cra (Olsén et al., 1993, Römling et al., 1998). CsgD itself modulates RpoS expression thereby indirectly affecting expression of other RpoS regulon target genes (Gualdi *et al.*, 2007). Cra induces biofilm formation in *E. coli* by activating curli synthesis. Transcription of the *csgDEFG* operon is regulated by Cra which binds to the promoter region (Reshamwala & Noronha, 2011).

Furthermore, various two-component systems (TCSs) affect curli gene expression. These systems constitute signaling pathways that regulate fundamental processes including cell differentiation, stress responses, and biofilm formation triggered by extrinsic stimuli (Majdalani & Gottesman, 2005, Gao & Stock, 2009). Curli synthesis in *E. coli* is under positive control of the EnvZ-OmpR two-component system (Vidal *et al.*, 1998, Prigent-Combaret *et al.*, 2001). The response regulator OmpR modulates curli gene expression in response to osmolarity by binding to the *csgD* promoter in *E. coli* and *S. typhimurium* (Jubelin *et al.*, 2005, Gerstel *et al.*, 2006). Moreover, the CpxAR (Dorel *et al.*, 1999) and the RcsCDB two-component systems (Vianney *et al.*, 2005) negatively regulate curli expression in *E. coli* (see chapter 1.4).

1.3.2 Cellulose polysaccharide fibrils

Cellulose is one of the most abundant organic macromolecules in nature as it constitutes the primary structural element of cell walls in higher plants (Ross *et al.*, 1991, Whitney & Howell, 2013). Beyond being produced by fungi, algae, and plants, cellulose was identified in *S. typhimurium* as the second principal constituent of the bacterial ECM (Zogaj *et al.*, 2001, Solano *et al.*, 2002). The investigation of further Enterobacteriaceae family members confirmed its presence in a variety of other species (Zogaj *et al.*, 2003).

The homopolymer cellulose is composed of covalently β-1,4-linked linear D-glucose monomer chains. Bundles of many glucan chains are aligned in the same orientation to form highly organized insoluble fibrils. This parallel arrangement confers considerable strength and resilience to the cellulose fibrils (Ross et al., 1991). The fluorescent dye Calcofluor White binds to cellulose and can therefore be used to visualize cellulose expressing bacteria in liquid culture or on solid medium (Zogaj et al., 2001, Solano et al., 2002).

Cellulose synthesis is mediated by the bacterial cellulose synthesis (*bcs*) locus comprising the operons *yhjRbcsQbcsABZC* and *bcsEFG* (Figure 2). The *bcs* genes appear to be transcribed constitutively in *S. typhimurium* and natural *E. coli* isolates (Zogaj et al., 2001). The *bcsA* gene codes for the catalytic subunit of cellulose synthase located in the inner membrane of the bacterial cell wall (Römling, 2002). It presumably catalyzes cellulose polymerization from the precursor molecule uridine diphosphate (UDP)-glucose and facilitates translocation of the

polymer across the inner membrane (Whitney & Howell, 2013, Ross et al., 1991). The BcsA protein possesses a specialized C-terminal domain designated PilZ which serves as binding domain for c-di-GMP. Cellulose synthesis is therefore linked to the second messenger which is central in controlling the switch between motility and sessility (discussed in the next chapter) (Amikam & Galperin, 2006, Ryjenkov *et al.*, 2006).

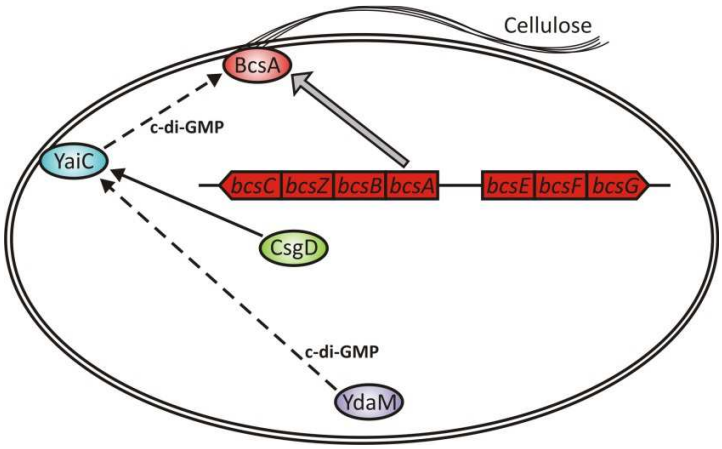


Figure 2: Regulation of cellulose synthesis in *E. coli*.
The FixJ/NarL-type transcription factor CsgD induces transcription of *yaiC*. Expression of YaiC is stimulated by the second messenger c-di-GMP produced by YdaM. BcsA, the catalytic subunit of cellulose synthase, is expressed from the *bcsABZC* operon. The activity of BcsA is stimulated by binding of c-di-GMP produced by YaiC leading to cellulose production.

1.3.3 A pivotal role for c-di-GMP

The bacterial second messenger c-di-GMP was discovered as an allosteric activator of cellulose biosynthesis in *Gluconacetobacter xylinus* (Ross *et al.*, 1987). c-di-GMP-mediated pathways are highly abundant signal transduction mechanisms in many bacteria (Jenal & Malone, 2006). It was shown that c-di-GMP stimulates transcription of the ECM master regulator *csgD* (Figure 1) and *yaiC* (Figure 2), encoding a c-di-GMP synthesizing enzyme itself, in *E. coli* (Weber *et al.*, 2006). The presence of c-di-GMP produced by YaiC is in turn compulsory for activation of BcsA and therefore for cellulose synthesis (Figure 2). Moreover, c-di-GMP constrains bacterial motility via binding to the PilZ domain of YcgR, a protein that interacts with motor proteins at the flagellar basal body in *E. coli* (Fang & Gomelsky, 2010, Boehm *et al.*, 2010). High c-di-GMP

levels further provoke cellulose accumulation in *Salmonella* which interferes with motility by impeding flagellar rotation through steric hindrance (Zorraquino *et al.*, 2013).

The turnover of c-di-GMP in bacteria is mainly regulated by enzymes harboring the GGDEF and EAL protein motifs, respectively (Tal *et al.*, 1998). These ubiquitous effector domains represent antagonists in c-di-GMP metabolism (Figure 3). The diguanylate cyclase (DGC) activity of the GGDEF motif is responsible for the synthesis of c-di-GMP from two GTP molecules (Ryjenkov *et al.*, 2005). EAL domain proteins act as phosphodiesterases (PDEs) that catalyze hydrolysis of c-di-GMP to linear pGpG (Schmidt *et al.*, 2005). GGDEF and EAL domain proteins represent two of the largest protein superfamilies of bacteria, especially in Gram-negative bacteria (Römling & Amikam, 2006).

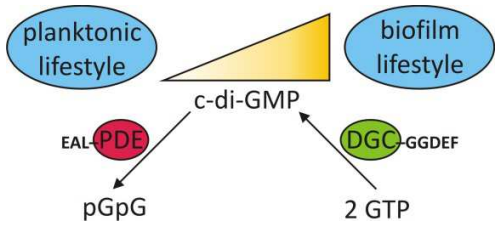


Figure 3: The transition between the two main bacterial lifestyles is ruled by c-di-GMP.
High c-di-GMP levels are a consequence of diguanylate cyclase (DGC) activity which results in biofilm formation. Low c-di-GMP levels are a consequence of phosphodiesterase (PDE) activity which results in the planktonic lifestyle.

Diguanylate cyclases and phosphodiesterases inversely regulate the transitions between sessility and motility and have opposite effects on several modes of multicellular behavior in *S. typhimurium* and *E. coli*. High c-di-GMP concentrations increase adherence to solid surfaces while low concentrations of c-di-GMP favor swarming and swimming (Simm *et al.*, 2004).

The importance of c-di-GMP signaling in the pathway regulating ECM production is emphasized by the fact that in *E. coli*, the two key DGCs YdaM and YaiC are expressed in a strict hierarchical manner thereby ensuring a chronologically defined sequence of events that culminates in biofilm formation (Weber et al., 2006) (Figure 4). Thus the regulatory cascade responsible for adhesion and biofilm formation is very fine-tuned and underlies a tight control which

guarantees spatially and temporally confined signaling. This quality is greatly reinforced by intensive signal relaying through small regulatory RNAs (discussed in the next chapter).

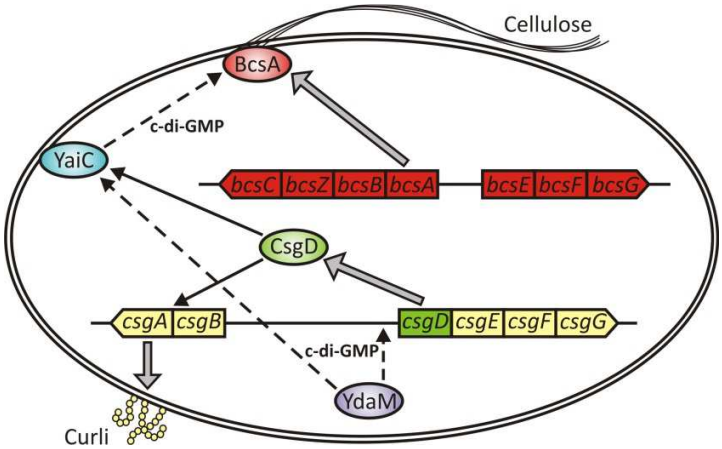


Figure 4: Integration of the different signal transduction pathways inducing biofilm formation in *E. coli*. The FixJ/NarL-type transcription factor CsgD and the second messenger c-di-GMP activate the expression of curli and cellulose, the two major components of the bacterial extracellular matrix.

1.3.4 Additional regulation provided by small regulatory RNAs

Besides the tremendous complexity of the dynamic interactions of transcription factors with the *csg* regulatory region on the DNA, the *csgD* mRNA acts as a counterpart on the RNA level through the action of small regulatory RNAs (sRNAs). sRNAs are non-coding RNAs that exert their regulatory function by annealing to target mRNAs (Waters & Storz, 2009). The RNA-binding protein Hfq aids in base-pairing of sRNAs to mRNAs by promoting sRNA stability and sRNA-mRNA duplex formation. Hfq-dependent sRNAs mostly impair translation or stability of their target mRNAs (Storz *et al.*, 2004).

The 5' untranslated region (UTR) of *csgD* mRNA serves as regulatory hub of post-transcriptional signal integration (Boehm & Vogel, 2012, Chambers & Sauer, 2013, Van Puyvelde *et al.*, 2013). In *E. coli*, *csgD* mRNA is repressed by five sRNAs that preferentially anneal upstream of the ribosome binding site (RBS) thereby interfering with translational initiation (Figure 5). Transcription of these sRNAs is triggered by various input signals such as cell surface stress or oxidative stress (Jørgensen *et al.*, 2012). For instance, induction of the sRNA RprA via the

RcsCDB two-component system results in repression of the ECM master regulator CsgD and of the diguanylate cyclase YdaM in *E. coli*. RprA base-pairs to the *csgD* and *ydaM* mRNAs in an Hfq-depending manner (Mika *et al.*, 2012).

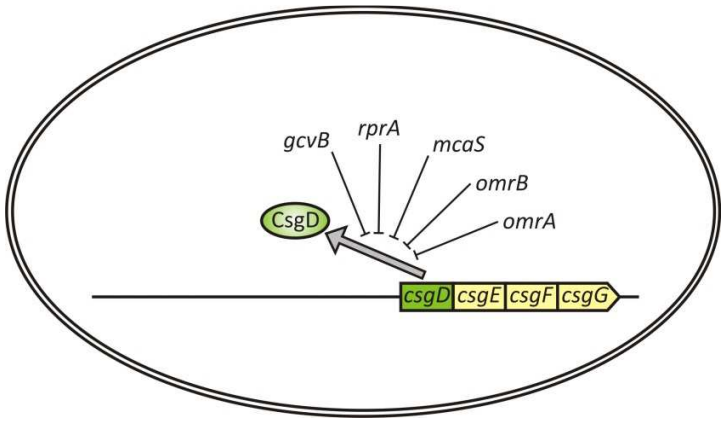


Figure 5: Expression of the FixJ/NarL-type transcription factor CsgD in *E. coli* is repressed by sRNAs. Translation of the *csgD* mRNA is repressed by the binding of at least five different sRNAs.

In conclusion, signal transduction inducing biofilm formation via the activation of the FixJ/NarL-type transcription factor CsgD is exceptionally complex. Activities of transcription factors, the second messenger c-di-GMP, and sRNA regulators are integrated at both transcriptional and post-transcriptional gene expression levels.

1.4 FixJ/NarL-type transcription factors of *Escherichia coli*

Transcription factors are proteins that are essential for regulating gene expression in prokaryotes and eukaryotes. In most cases, these proteins act by binding to regulatory regions on the DNA thereby affecting gene transcription. In this way the interplay of various transcription factors largely determines the cellular fate.

The FixJ/NarL family of transcriptional factors comprises 19 members in *E. coli* K-12 (www.uniprot.org). Most of them are similar in size with a length of about 200 amino acids. FixJ/NarL-type transcription factors can be classified into three groups according to their domain structure (Table 1). The largest group consists of ten proteins that harbor a C-terminal DNA-binding domain with a helix-turn-helix (HTH) LuxR-type motif (Henikoff *et al.*, 1990). The ECM master regulator CsgD (see chapter 1.3.1) and YjjQ, whose function has been addressed in this work, belong to this group of proteins. In the eight proteins of the second group, the N-terminus constitutes a receiver domain. Phosphorylation of a conserved aspartate residue in the receiver domain enables these proteins to act as signal transducers in two-component systems (TCSs) (Gao *et al.*, 2007, Gao & Stock, 2009). A well-studied representative of the second group is the response regulator RcsB (Majdalani & Gottesman, 2005). The third group consists of only one member, YahA, which is composed of an N-terminal DNA-binding domain with a HTH LuxR-type motif and a C-terminal EAL domain responsible for c-di-GMP degradation (Schmidt et al., 2005) (see chapter 1.3.3).

Oligomerization of regulatory proteins is a common feature of many signaling pathways involved in diverse cellular processes. Within the *E. coli* K-12 FixJ/NarL-type transcription factor family, RcsB is known to homodimerize in order to exert its regulatory functions (Majdalani *et al.*, 2002, Schwan *et al.*, 2007). Furthermore, some FixJ/NarL-type transcription factors heterodimerize with RcsB to regulate expression of genes distinct to the ones targeted by homodimers (Vianney et al., 2005, Francez-Charlot *et al.*, 2003). This combinatorial diversity amplifies the possibility to modulate transcription of numerous genes with a comparably small repertoire of transcriptional regulators. In addition, heterodimers can act as activators in some cases but can also behave as repressors under certain circumstances. This specificity adds

another level of complexity to the sophisticated gene regulation pathways provided by FixJ/NarL-type transcription factors (Figure 6).

The best-studied candidate for dimerization within the FixJ/NarL-type transcription factor family is the response regulator RcsB. It represents the key player of the RcsCDB TCS and is conserved in Enterobacteriaceae (Huang *et al.*, 2006). In *E. coli*, RcsB homodimers activate transcription of the small regulatory RNA RprA that stimulates translation of the general stress sigma factor RpoS (σ^{38}) (Majdalani et al., 2002, Battesti *et al.*, 2011) and inhibits biofilm formation through an increase in RpoS levels and repression of CsgD (Mika et al., 2012, Ferrières *et al.*, 2009). RcsB positively controls the expression of type 1 fimbriae in *E. coli* (Schwan et al., 2007) (see chapter 5.3) and of Mat fimbriae in neonatal meningitis-causing *E. coli* (Lehti *et al.*, 2012b). Fimbrial adhesin proteins are important factors in bacterial virulence and biofilm formation (Van Houdt & Michiels, 2005, Klemm *et al.*, 2010).

For the regulation of other target genes, it is known that RcsB cooperates with the unstable auxiliary protein RcsA. RcsB/RcsA heterodimers activate transcription of the *wca/cps* genes responsible for capsular polysaccharide synthesis in *E. coli* (Stout *et al.*, 1991). The *wca* locus, formerly called *cps*, constitutes a putative biofilm determinant (Danese *et al.*, 2000). Furthermore, they activate transcription of the *yjbEFGH* operon which is also involved in production of exopolysaccharides and affects colony morphology in *E. coli* (Ferrières *et al.*, 2007). In contrast, RcsB/RcsA heterodimers negatively regulate transcription of *flhDC* coding for the master regulator of flagellar motility in *E. coli* (Francez-Charlot et al., 2003). Moreover, the *csgBA* and *csgDEFG* operons are repressed in *E. coli* by RcsB/RcsA heterodimers resulting in inhibited curli synthesis and adherence (Vianney et al., 2005, Ferrières & Clarke, 2003, Carter *et al.*, 2012) (see chapter 1.3.1).

In *E. coli*, RcsB/BglJ heterodimers activate expression of the pleiotropic regulator LeuO (Stratmann *et al.*, 2012) as well as of the *bgl* (aryl-β,D-glucoside) operon by relieving H-NS-mediated repression (Venkatesh *et al.*, 2010). Recently, Castanié-Cornet and coworkers reported the formation of RcsB/GadE heterodimers in *E. coli* under acid stress conditions (Castanié-Cornet *et al.*, 2010).

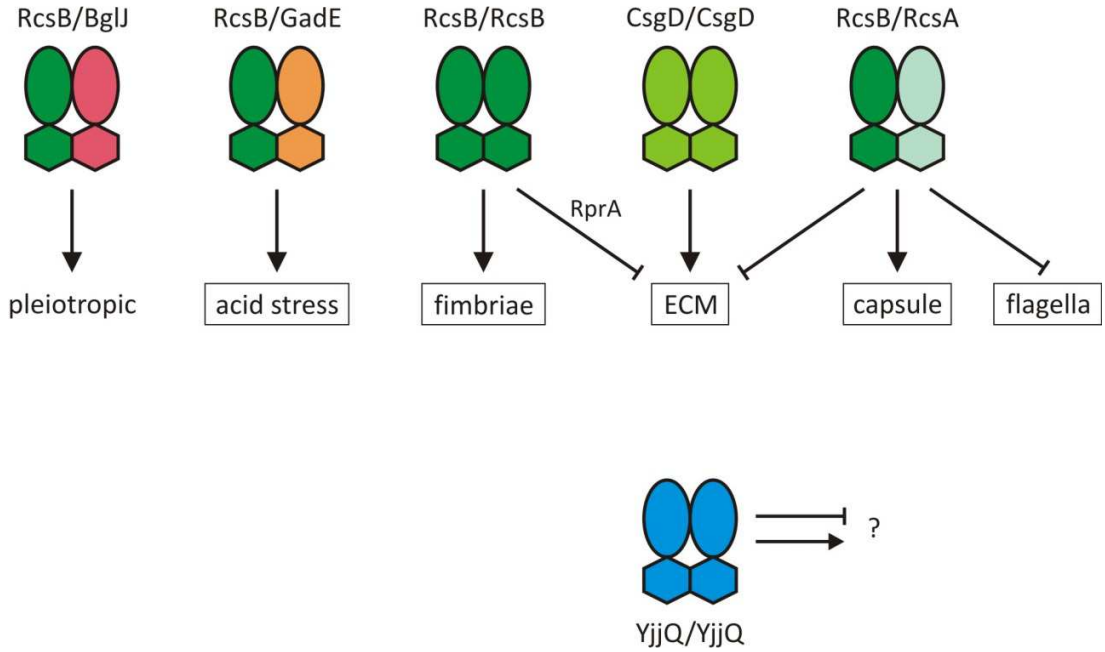


Figure 6: Regulation of cellular pathways by FixJ/NarL-type transcription factors in *E. coli*.
RcsB/BglJ heterodimers activate expression of the *bgl* operon responsible for alternative carbon source utilization and of the pleiotropic regulator LeuO. RcsB/GadE heterodimers mediate acid stress responses. RcsB homodimers promote adhesion via stimulation of the formation of type 1 fimbriae. In contrast, RcsB homodimers suppress biofilm formation via repression of CsgD by the sRNA RprA. The ECM master regulator CsgD promotes curli expression and cellulose synthesis. RcsB/RcsA heterodimers suppress biofilm formation via repression of curli expression. Moreover, RcsB/RcsA heterodimers promote capsule formation via stimulation of the synthesis of capsular polysaccharides. RcsB/RcsA heterodimers suppress flagellar motility via repression of the master regulator of flagellar motility. Targets of YjjQ are unknown.

The LexA-based bacterial two-hybrid system (Dmitrova *et al.*, 1998) was used previously in our laboratory to analyze RcsB heterodimerization properties with the other 18 FixJ/NarL-type transcription factors in *E. coli* K-12 (laboratory data, unpublished) (see chapter 2.1.3). The same method was applied to investigate homodimerization of all FixJ/NarL-type transcription factors in *E. coli* K-12. The results from the β-galactosidase assays indicated that YjjQ forms homodimers as well as weak heterodimers with RcsB under the conditions tested (Table 1). Furthermore, the findings suggested that CsgD forms a homodimer under the conditions tested.

Table 1: Dimerization properties and domain structure of the FixJ/NarL-type transcription factors in *E. coli* K-12.

HTH LuxR-type proteins	Length (amino acids)	TCS	Homodimer formation[1]	Heterodimer formation with RcsB[1]	Domain composition
YjjQ	241	-	+	+/-	
BglJ	225	-	-	+	
CsgD	216	-	+	-	
MalT	901	-	+	-	
SdiA	240	-	+	-	
YhjB	200	(predicted)	+	-	
DctR	176	-	-	+	
GadE	175	-	-	+	
MatA	196	-	-	+	
RcsA	207	-	-	+	
EvgA	204	EvgSA	+	+	
FimZ	210	-	+	+/-	
NarL	216	NarQX	+	+/-	
NarP	215	NarQX	+	+/-	
RcsB	216	RcsCDB	+	n.a.	
UhpA	196	UhpBA	+	+/-	
UvrY	218	BarA-UvrY	+	+/-	
YgeK	210	(predicted)	-	-	
YahA	362	-	+	+/-	

[1] determined with the LexA-based bacterial two-hybrid system (Table 2) (laboratory data, unpublished).
+: strong interaction; -: no detectable interaction; ?: weak interaction.
HTH LuxR-type motif; Response regulatory motif (receiver domain); EAL motif

1.5 The FixJ/NarL-type transcription factor YjjQ

YjjQ is a transcriptional regulator conserved in commensal and several pathogenic bacteria such as enterohaemorrhagic *E. coli* (EHEC) and uropathogenic *E. coli* (UPEC). Transcription of *yjjQ* was reported to be induced in M9 minimal medium (Li *et al.*, 2008). Mutation of *yjjQ* was shown to attenuate virulence of avian pathogenic *E. coli* (APEC) possibly due to impaired iron uptake (Li et al., 2008). In a screen for genes involved in the repression of motility in UPEC strain CFT073 constitutively expressing type 1 fimbriae, *yjjQ* emerged as putative candidate

(Simms & Mobley, 2008). Transposon mutagenesis performed on a phase-locked ON fimbriation variant (see chapter 5.3) of CFT073 followed by a screen for mutants with restored swimming motility revealed a disruption in *yjjQ* due to a Tn5 insertion. This finding implies that YjjQ may impinge upon bacterial motility (Simms & Mobley, 2008). However, the putative role of this transcription factor in motility was not further analyzed.

In *E. coli*, YjjQ is encoded in an operon together with BglJ, another member of the FixJ/NarL-type transcription factor family (Figure 7). Adjacent to the *yjjQ-bglJ* operon, the *yjjP* gene is situated coding for a membrane protein of unknown function. The *yjjP-yjjQ-bglJ* locus is silenced by the abundant histone-like nucleoid-associated protein H-NS (Stratmann *et al.*, 2008). H-NS greatly contributes to the organization and compaction of bacterial chromatin thereby maintaining nucleoid structure (Ali *et al.*, 2013). But beyond that H-NS also acts as a global repressor that affects transcription of about 5% of the *E. coli* genes (Hommais *et al.*, 2001, Dorman, 2004). For that reason YjjQ is assumed not to be expressed under laboratory conditions (Stratmann et al., 2008).

In addition to H-NS-mediated regulation, expression of the *yjjQ-bglJ* operon in *E. coli* underlies regulation by the LysR-type transcription factor LeuO. This protein is a pleiotropic regulator of multiple loci including genes related to the stress response and pathogenicity of Enterobacteriaceae (Shimada *et al.*, 2011a, Stratmann et al., 2012). LeuO expression is repressed by H-NS (Klauck *et al.*, 1997, Chen *et al.*, 2001) and is increased under nutrient starved conditions (Majumder *et al.*, 2001) as well as during transition into stationary growth phase (Fang *et al.*, 2000). It modulates transcription of *yjjP* lying upstream of *yjjQ-bglJ* in divergent orientation. Furthermore, LeuO is a transcriptional activator and putative H-NS antagonist at the *yjjQ-bglJ* operon (Stratmann et al., 2008) (Figure 7). At this locus, LeuO may compete with H-NS for DNA binding in the promoter region. Expression of *yjjQ-bglJ* will be activated depending on the outcome of this competition.

BglJ is able to heterodimerize with the FixJ/NarL transcription factor RcsB. As stated before, RcsB/BglJ heterodimers activate transcription of *leuO* in *E. coli* (Stratmann et al., 2012) (see chapter 1.4). In this way this regulatory circuit constitutes a double positive feedback loop.

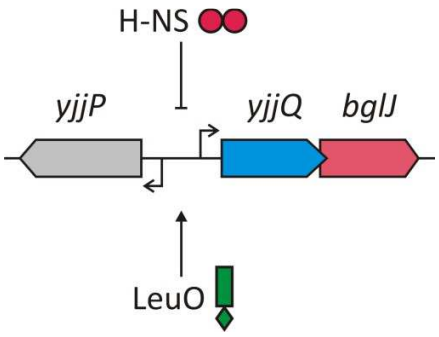


Figure 7: Regulation of the *yjjQ-bglJ* promoter region in *E. coli*.
The nucleoid-associated protein H-NS represses transcription of *yjjQ* and *bglJ* via binding to the intergenic region of *YjjP* and the *yjjQ-bglJ* operon. In contrast, the LysR-type transcription factor LeuO induces transcription of the *yjjQ-bglJ* operon.

Microarray analysis performed in *E. coli* K-12 and UPEC strain CFT073 revealed that YjjQ overexpression results in significant downregulation of more than 20 loci partly associated with pathogenicity and biofilm formation (laboratory data, unpublished). One of the loci substantially repressed by YjjQ is the *flhDC* locus encoding the master regulator of flagellar motility (see chapter 5.3). Another YjjQ target is the *bcs* locus mediating cellulose synthesis in bacteria (Table 3). This exopolysaccharide is known to play a fundamental role in biofilm formation and adhesion processes (Zogaj et al., 2001, Saldaña *et al.*, 2009). A detailed description of this locus can be found in chapter 1.3.2. Therefore, the transcription factor YjjQ may be involved in regulating motility as well as biofilm formation and adhesion in Enterobacteriaceae.

1.6 Aim of this thesis

So far, the role of the FixJ/NarL-type transcription factor YjjQ in gene regulation remains elusive. Putative target genes of YjjQ in *E. coli* were identified in a microarray, but not further characterized, and the status of YjjQ in signal transduction pathways remains to be established. In this study, the role of YjjQ is investigated in *E. coli* K-12 and the UPEC strains CFT073 and 536. Since only very little is known about this protein the aim is to figure out novel functions of YjjQ.

2. Results

2.1 Characterization of dimerization properties between FixJ/NarL-type transcription factors in *E. coli*

Within the FixJ/NarL-type transcription factor family of *E. coli* K-12, some members are known to homodimerize and/or heterodimerize in order to exert their regulatory functions (Majdalani & Gottesman, 2005) (see chapter 1.4). In our laboratory the interaction of the response regulator RcsB with other FixJ/NarL-type transcription factors present in *E. coli* K-12 was already analyzed (Venkatesh et al., 2010). But additional and so far unknown interactions between further FixJ/NarL-type transcription factors may occur. Within the framework of my project I investigated whether more dimer pairs exist. To characterize the dimerization properties between different FixJ/NarL-type transcription factors in *E. coli* K-12, bacterial two-hybrid assays were performed.

2.1.1 The "Bacterial Adenylate Cyclase Two-Hybrid" (BACTH) system

Initially I used the BACTH system (Karimova *et al.*, 1998) to examine dimerization of hybrid FixJ/NarL-type transcription factors. This method is based on the interaction-mediated reconstitution of enzyme activity. Adenylate cyclase is responsible for the synthesis of the second messenger 3',5'-cyclic adenosine monophosphate (cAMP). The catalytic domain of adenylate cyclase from *Bordetella pertussis* encoded by *cyaA* consists of two complementary fragments, T18 and T25 (Figure 8). These two fragments are fused to the proteins of interest and expressed in a reporter strain lacking endogenous adenylate cyclase activity (*cyaA*$^-$). Heterodimerization of the hybrid proteins results in functional complementation between T18 and T25 and, consequently, in cAMP synthesis. cAMP binds to the cAMP receptor protein (CRP; also termed CAP for catabolite activator protein) generating a cAMP/CRP complex that activates transcription of catabolic operons such as *lac* and *mal*. Thus, the bacteria become able to utilize lactose or maltose and accordingly exhibit Lac$^+$ or Mal$^+$ phenotypes on the respective indicator plates. Via this mechanism the interaction of proteins can be distinguished phenotypically on two types of growth medium (Karimova et al., 1998) (Figure 8).

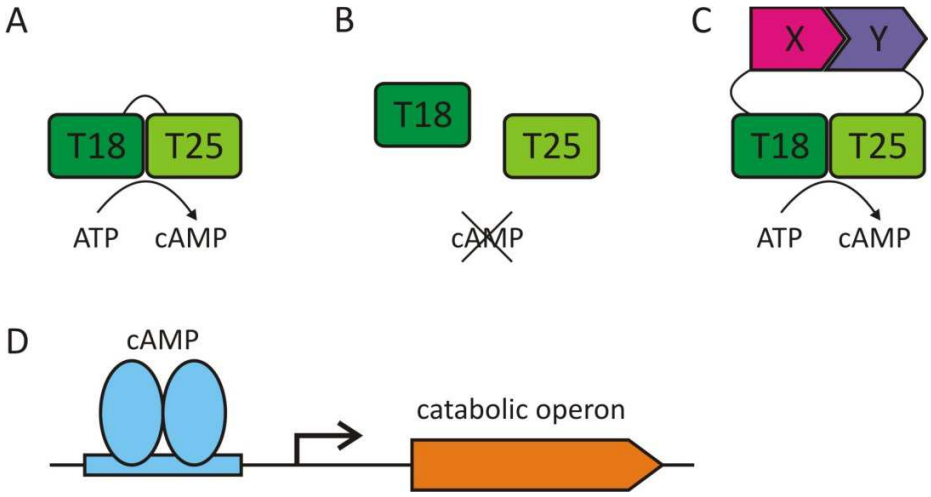


Figure 8: Principle of the bacterial adenylate cyclase two-hybrid (BACTH) system.
In the BACTH system the *E. coli* strain BTH101 (*cyaA*$^-$) is used. (A) Interaction of the T18 and T25 fragments of the adenylate cyclase catalytic domain induces synthesis of the second messenger cAMP. (B) The catalytic domain of adenylate cyclase is not active when T18 and T25 are physically separated. (C) Interaction between the hybrid proteins via dimerization of X and Y results in restored catalytic activity and consequently cAMP synthesis. (D) cAMP forms a complex with the dimeric transcription factor CRP, depicted in blue, which binds to the DNA-binding site for the cAMP/CRP complex represented by the blue box and initiates transcription of the reporter genes (Karimova et al., 1998).

Eight FixJ/NarL-type transcription factors were chosen for the BACTH assays: BglJ, DctR, EvgA, GadE, MatA, RcsA, RcsB, and YjjQ. This selection includes EvgA known to be a global response regulator in the EvgAS TCS (Nishino *et al.*, 2003), three proteins that our group is interested in (BglJ, RcsB, and YjjQ), and four proteins that were shown to be unable to form homodimers (DctR, GadE, MatA, and RcsA) (laboratory data, unpublished) (Table 1). Each gene was cloned into the plasmids pUT18C and pKT25. The resulting plasmids pKEKD1-16 encoded fusions of the proteins of interest to the C-termini of T18 and T25 (T18-X and T25-Y) each under the control of an IPTG-inducible lac promoter, respectively (Table 13). The *rcsA* and *rcsB* genes were additionally cloned into the plasmids pUT18 and p25-N. The resulting plasmids pKEKD17-20 encoded fusions of RcsA and RcsB to the N-termini of T18 and T25 (X-T18 and Y-T25) each under the control of an IPTG-inducible lac promoter, respectively (Table 13). Interaction of hybrid leucine zippers encoded by the plasmids pUT18C-zip and pKT25-zip was measured as positive control (Karimova et al., 1998). Since it is known that RcsB forms homodimers as well as heterodimers with RcsA (Majdalani & Gottesman, 2005) (see chapter 1.4), these

combinations were used as additional control for the functionality of the system. The hybrid proteins were co-expressed in the adenylate cyclase (*cyaA*)-deficient *E. coli* strain BTH101. If X and Y interact, T18 and T25 should catalyze cAMP synthesis that is in turn manifested as transcriptional activation of the catabolic *lac* and *mal* reporter operons.

In *E. coli*, expression of *lacZ* encoding β-galactosidase is positively controlled by cAMP/CRP (Kolb *et al.*, 1993). Cleavage of the chromogenic lactose analogue X-gal by β-galactosidase leads to the formation of a blue product. Hence, bacteria expressing interacting hybrid proteins form blue colonies on LB agar plates in the presence X-gal, while cells expressing non-interacting proteins remain white. The maltose regulon is induced by the FixJ/NarL-type transcription factor MalT. Expression of MalT in *E. coli* is likewise controlled by cAMP/CRP (Chapon & Kolb, 1983) and thus adenylate cyclase-deficient bacteria are unable to utilize maltose. Therefore they form white colonies on MacConkey agar plates containing maltose, while adenylate cyclase-proficient bacteria form red colonies indicating successful complementation of the catalytic domain. For this reason there is a direct correlation between cAMP synthesis and the phenotype of bacterial colonies on these kinds of indicator media.

The corresponding recombinant plasmids were transformed into *E. coli* strain BTH101 (*cyaA*$^-$) leading to co-expression of the T18 and T25 hybrid proteins. BTH101 transformant strains were selected overnight either on LB X-gal agar plates with the appropriate antibiotics at 28°C, or on maltose MacConkey agar plates with the appropriate antibiotics at 37°C. To obtain phenotypes on LB X-gal agar plates, protein expression from the plasmids required induction by IPTG. For that reason this growth medium was supplemented with 0.2 mM IPTG. On maltose MacConkey agar plates, the presence of IPTG did not make a difference for the phenotype. As expected, transformants of the positive control expressing the T18-zip and T25-zip hybrid proteins displayed Lac$^+$ and Mal$^+$ phenotypes (blue and red colonies) whereas transformants of the negative control expressing T18 and T25 displayed Lac$^-$ and Mal$^-$ phenotypes (white colonies) on the respective indicator plates (Figure 9). For most of the tested combinations of hybrid FixJ/NarL-type transcription factors, the BACTH system yielded Lac$^-$ and Mal$^-$ phenotypes. Although RcsB is known to homodimerize (Majdalani & Gottesman, 2005), even co-expression of RcsB-T18 and RcsB-T25 resulted in only scattered blue or red staining with the vast majority

of colonies being white (Figure 9). It seems that the BACTH system is not sensitive enough to analyze interactions between FixJ/NarL-type transcription factors. Since no further dimer pairs could be detected irrespective of the growth conditions, I tested another bacterial two-hybrid system using a quantitative approach in form of the β-galactosidase assay.

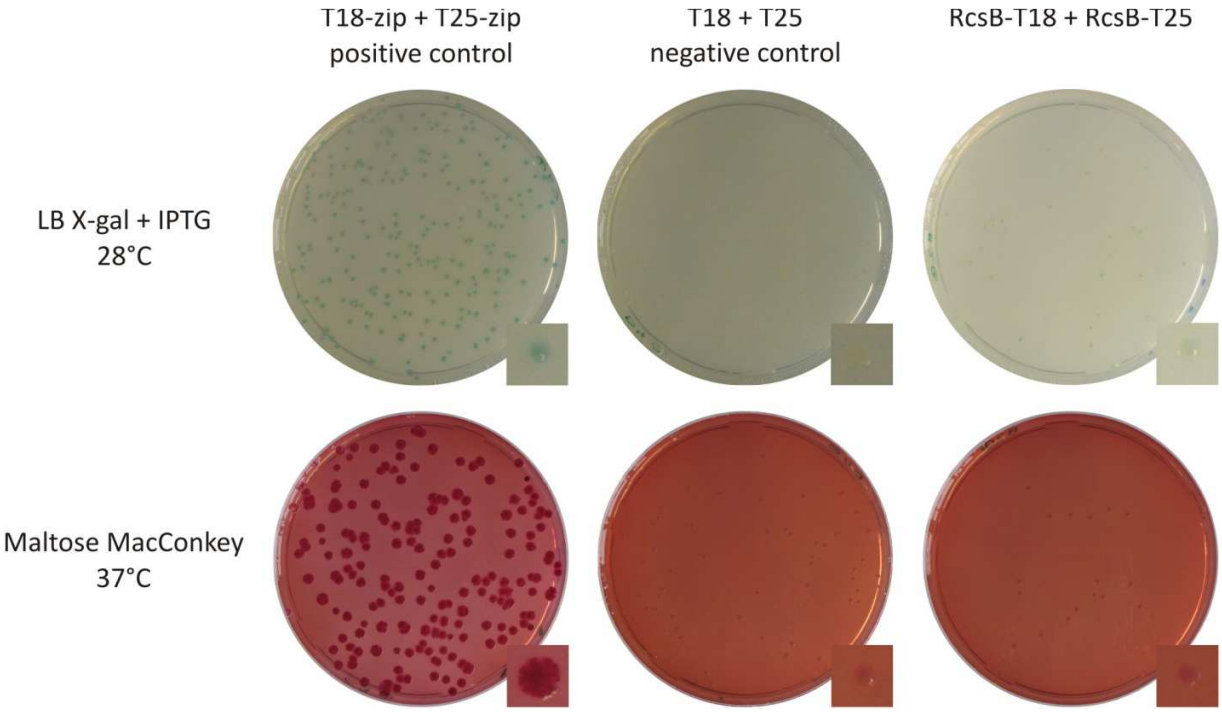


Figure 9: Homodimerization of RcsB-T18 and RcsB-T25 hybrid proteins analyzed with the BACTH system in *E. coli*.
RcsB homodimerization was examined using the BACTH system by co-expression of the hybrid proteins in the reporter strain BTH101 (*cyaA*$^-$) grown on two types of indicator media. Co-expression of T18-zip with T25-zip (positive control) causes Lac$^+$ and Mal$^+$ phenotypes, respectively. Co-expression of T18 with T25 (negative control) causes Lac$^-$ and Mal$^-$ phenotypes, respectively. Co-expression of RcsB-T18 with RcsB-T25 causes Lac$^-$ and Mal$^-$ phenotypes as well, respectively. The following plasmids were used: pKT25-zip and pUT18-zip for the positive control, p25-N and pUT18 for the negative control, p25-N-RcsB (pKEKD20) and pUT18-RcsB (pKEKD18) for homodimerization analysis. Phenotypes as observed after 2 days of growth on LB agar plates supplemented with 40 µg/ml X-gal and 0.2 mM IPTG at 28°C or on MacConkey plates supplemented with 1% maltose at 37°C.

2.1.2 The cI-α bacterial two-hybrid system

The cI-α bacterial two-hybrid system (Dove & Hochschild, 2004) is based on the activation of a transcription initiation complex. One of the proteins of interest is tethered to DNA by the DNA-binding domain of the phage λ repressor cI (λcI) (Figure 10). The other one is linked to the N-terminal domain of the alpha subunit (α-NTD) of RNA polymerase. The promoter used in this method contains a binding site for the λcI repressor in its upstream regulatory region. Interaction between the DNA-bound fusion protein and the RNA polymerase subunit fusion protein stabilizes the binding of RNA polymerase to the promoter, thus activating transcription of a *lacZ* reporter. With this system protein interaction can be measured quantitatively in a β-galactosidase assay.

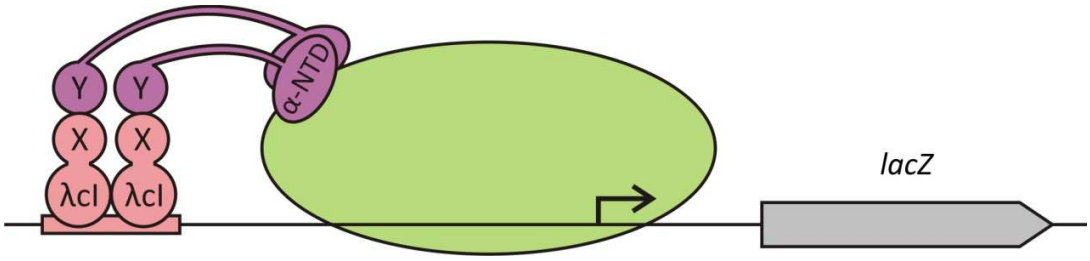


Figure 10: Principle of the cI-α bacterial two-hybrid system.
In the cI-α bacterial two-hybrid system, the α-NTD-Y fusion protein, depicted in purple, assembles with RNA polymerase, depicted in green. The dimeric λcI-X fusion protein, depicted in pink, binds to the DNA-binding site for the λcI repressor represented by the pink box. Interaction between the hybrid proteins via dimerization of X and Y leads to the formation of a transcription initiation complex on the promoter that induces expression of the reporter gene (Dove & Hochschild, 2004).

In order to test the suitability of the cI-α bacterial two-hybrid system I ran a test for homodimerization of RcsB hybrid proteins (Figure 11). For that reason the *rcsB* gene was cloned into the plasmids pAC λcI-β 831-1057 and pBR α-β 831-1057. The resulting plasmids pKEKD23 and pKEKD24 encoded fusions of RcsB to the C-termini of λcI and α-NTD each under the control of an IPTG-inducible lacUV5 promoter, respectively (Table 13). Positive control plasmids pAC λcI-β 831-1057 and pBR α-σ^{70} D581G expressed the interacting proteins β 831-1057 and σ^{70} D581G. Negative control plasmids pAC λcI and pBR α expressed the non-interacting proteins λcI and α-NTD. The hybrid proteins are co-expressed in *E. coli* strain FW102 carrying the *lacZ*

reporter on the F'-plasmid under the control of an IPTG-inducible lacUV5 promoter. If λcI-RcsB and α-NTD-RcsB interact, the initiation of transcription at the hybrid promoter should result in *lacZ* expression that is in turn manifested as β-galactosidase synthesis.

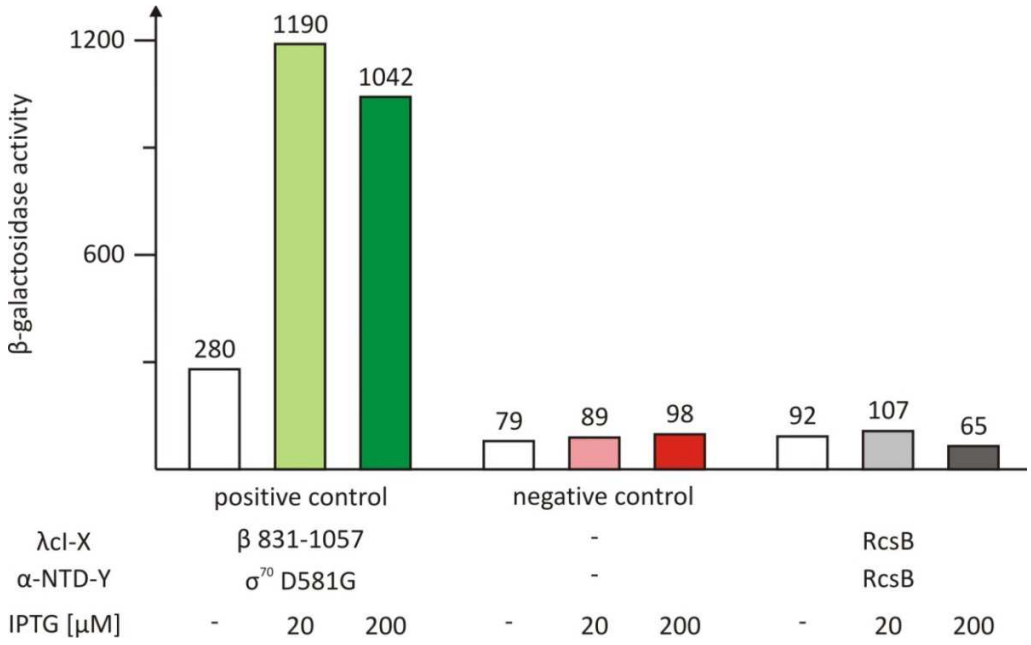


Figure 11: Homodimerization of λcI-RcsB and α-NTD-RcsB hybrid proteins analyzed with the cI-α bacterial two-hybrid system in *E. coli*.
RcsB homodimerization was examined using the cI-α bacterial two-hybrid system by co-expression of the hybrid proteins in the *lacZ* reporter strain FW102 followed by β-galactosidase assays. Co-expression of λcI-β 831-1057 and α-σ^{70} D581G (positive control) results in elevated β-galactosidase activities in an IPTG-dependent manner. Co-expression of λcI and α-NTD (negative control) only generates basal level β-galactosidase activities. Co-expression of λcI-RcsB and α-NTD-RcsB also generates basal level β-galactosidase activities. The following plasmids were used: pAC λcI-β 831-1057 and pBR α-σ^{70} D581G for the positive control, pAC λcI and pBR α for the negative control, λcI-RcsB (pKEKD23) and α-NTD-RcsB (pKEKD24) for homodimerization analysis. Miller unit values represent averages calculated from at least two independent experiments. Cultures for β-galactosidase assays were grown overnight in LB with antibiotics +/- IPTG at 37°C; exponential cultures were inoculated from the overnight cultures to an OD_{600} of 0.05 and grown with antibiotics +/- IPTG to an OD_{600} of approx. 0.5 at 37°C.

The corresponding recombinant plasmids were transformed into *E. coli* strain FW102 leading to co-expression of the hybrid proteins induced by two different concentrations of IPTG (Figure 11). FW102 transformants of the positive control expressing β 831-1057 and σ^{70} D581G exhibit increased β-galactosidase activities dependent on IPTG (Figure 11). Transformants of the

negative control expressing λcI and α-NTD exhibit only basal level β-galactosidase activities independent of IPTG. Co-expression of λcI-RcsB and α-NTD-RcsB also generates basal level β-galactosidase activities irrespective of the IPTG concentration. The RcsB transformants did not provide elevated β-galactosidase activities compared to the negative control (Figure 11). Thus, no RcsB homodimerization could be detected using the cI-α bacterial two-hybrid system under the growth conditions applied. For that reason I continued with the LexA-based bacterial two-hybrid system.

2.1.3 The LexA-based bacterial two-hybrid system

The LexA-based bacterial two-hybrid system (Dmitrova et al., 1998) is based on the interaction-mediated reconstitution of a functional repressor. The method specifically detects heterodimerization of proteins by the use of two derivatives of the dimeric LexA repressor with different DNA binding specificities. The two proteins of interest are fused to a wild-type and a mutant LexA DNA-binding domain consisting of the N-terminal amino acids 1 to 87 (Figure 12). Their association is measured by the repression of a sulA promoter-*lacZ* reporter gene fusion with a hybrid operator containing a wild-type half-site and a mutated half-site. The assay thus allows monitoring selectively the interaction of two heterologous proteins even if one or both partners are able to form homodimers.

The LexA two-hybrid system was successfully used for interaction analysis of RcsB with other FixJ/NarL-type transcription factors in *E. coli* K-12 (Venkatesh et al., 2010) (Table 1). But it has the disadvantage that interaction of proteins is reflected as repression of the *lacZ* reporter. Therefore the proteins need to be expressed at high levels which in some cases results in reduced bacterial growth.

For the wild-type LexA constructs I used the same eight FixJ/NarL-type transcription factors that have been chosen for the BACTH assays: BglJ, DctR, EvgA, GadE, MatA, RcsA, RcsB, and YjjQ. The corresponding genes were cloned by former laboratory members. For the mutant LexA constructs only RcsB, BglJ, EvgA, and GadE were used. Plasmids harboring *rcsB* and *bglJ* were already available. The remaining genes *evgA* and *gadE* were cloned into the plasmid pKES189. The resulting plasmids pKEKD21 and pKEKD22 encoded fusions of EvgA and GadE to the C-

terminus of the mutant $LexA_{(1-87)408}$ DNA-binding domain each under the control of an IPTG-inducible lacUV5 promoter, respectively (Table 13).

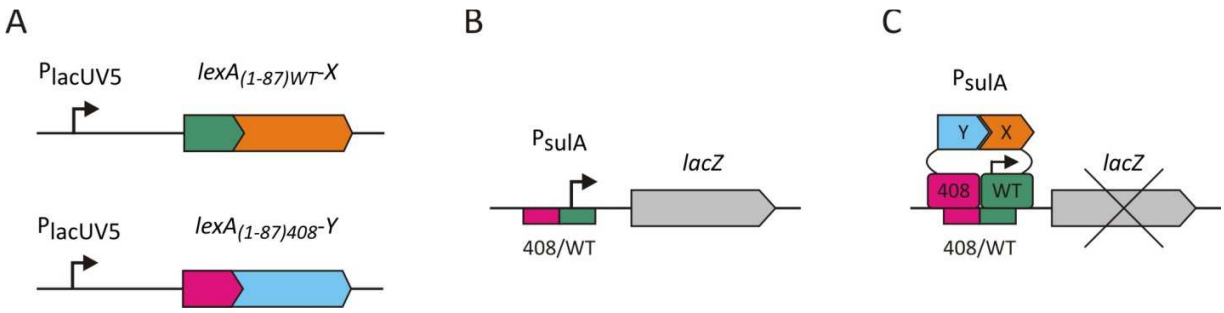


Figure 12: Principle of the LexA-based bacterial two-hybrid system.
(A) In the LexA-based bacterial two-hybrid system, the two proteins of interest X and Y are fused to LexA derivatives (WT and 408) that are able to dimerize but possess different DNA binding specificities. (B) In the absence of heterodimers, the reporter gene is transcribed since the hybrid *lexA* operator represented by the colored boxes is not occupied. (C) Interaction between the fusion proteins via heterodimerization of X and Y leads to the formation of a functional dimeric repressor, which binds to the hybrid *lexA* operator and blocks the sulA promoter thereby repressing transcription of the reporter gene (Dmitrova et al., 1998).

Interaction of $LexA_{(1-87)WT}$-Fos and $LexA_{(1-87)408}$-Jun encoded by the plasmids pMS604 and pDP804, measured before (Venkatesh et al., 2010), was used as positive control reference value. The corresponding recombinant plasmids were transformed into *E. coli* strain S3440 (Δ*rcsB*) leading to co-expression of $LexA_{(1-87)WT}$-X and $LexA_{(1-87)408}$-Y induced by IPTG. If X and Y interact, the dimeric repressor becomes functional and should bind to the hybrid *lexA* operator thereby blocking the sulA promoter. This binding should lead to a repression of *lacZ* reporter transcription that is in turn manifested as reduced β-galactosidase activities.
The results of the experiments are summarized in Figure 13.

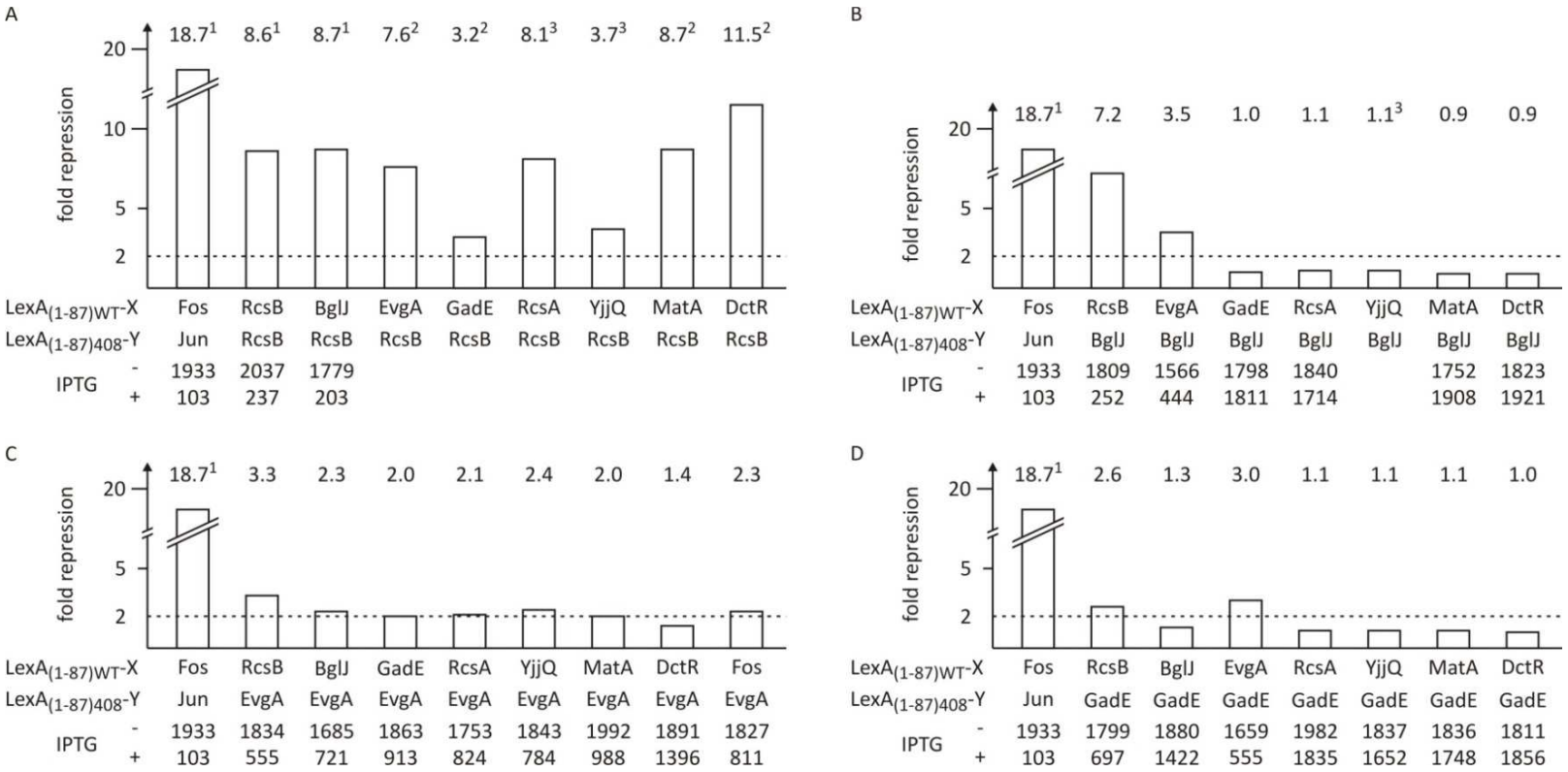


Figure 13: Dimerization analysis of selected hybrid FixJ/NarL-type transcription factors using the LexA-based bacterial two-hybrid system in *E. coli*. Dimerization of (A) RcsB, (B) BglJ, (C) EvgA, and (D) GadE with other FixJ/NarL-type transcription factors was examined using the LexA-based bacterial two-hybrid system by co-expression of the hybrid proteins in the *lacZ* reporter strain S3440 (Δ*rcsB*) followed by β-galactosidase assays. Fold repression values of β-galactosidase activities (indicated on top) were calculated from the averages of uninduced versus IPTG-induced samples from at least two independent experiments (indicated on the bottom). Fold repression values labelled with 1 represent interactions that were shown previously in *E. coli* strain S3442 (Δ*rcsB* Δ(*yjjP-yjjQ-bglJ*)) (Venkatesh et al., 2010). Fold repression values labelled with 2 represent interactions that were shown previously (laboratory data, unpublished). Fold repression values labelled with 3 represent interactions that were shown previously in *E. coli* strain S3442 (laboratory data, unpublished). The following plasmids were used: $LexA_{(1-87)WT}$-Fos (pMS604), $LexA_{(1-87)WT}$-RcsB (pKEMK17), $LexA_{(1-87)WT}$-BglJ (pKEAP30), $LexA_{(1-87)WT}$-EvgA (pKEMK15), $LexA_{(1-87)WT}$-GadE (pKEMK16), $LexA_{(1-87)WT}$-RcsA (pKES192), $LexA_{(1-87)WT}$-YjjQ (pKEAP27), $LexA_{(1-87)WT}$-MatA (pKEMK4), $LexA_{(1-87)WT}$-DctR (pKEMK1), $LexA_{(1-87)408}$-Jun (pDP804), $LexA_{(1-87)408}$-RcsB (pKEAP28), $LexA_{(1-87)408}$-BglJ (pKEAP29), $LexA_{(1-87)408}$-EvgA (pKEKD21), $LexA_{(1-87)408}$-GadE (pKEKD22). Cultures for β-galactosidase assays were grown overnight in LB with antibiotics +/- 1 mM IPTG at 37°C; exponential cultures were inoculated from the overnight cultures to an OD_{600} of 0.05 and grown with antibiotics +/- 1 mM IPTG to an OD_{600} of approx. 0.5 at 37°C.

As shown in Table 2, I measured a 7.2-fold repression of reporter gene expression by RcsB/BglJ heterodimers which confirms previous data (Venkatesh et al., 2010, Stratmann et al., 2012). Although an interaction of RcsB with GadE is already published (Castanié-Cornet et al., 2010), the proteins seem to form only weak heterodimers with fold-changes of 3.2 and 2.6. Most of the other heterodimer combinations repressed the system only to a minor extent (0.9- to 1.3-fold). BglJ and GadE do not interact since both combinations, $LexA_{(1\text{-}87)WT}$-BglJ with $LexA_{(1\text{-}87)408}$-GadE and $LexA_{(1\text{-}87)WT}$-GadE with $LexA_{(1\text{-}87)408}$-BglJ, did only cause a small fold-change in repression. Furthermore, both proteins did not interact with RcsA, YjjQ, MatA, and DctR.

Strikingly, nearly all repression levels generated with EvgA, either with the wild-type or the mutated 408 fusion, are higher than the ones of the other fusion proteins (1.4- to 3.5-fold) (Table 2). In order to find out if this phenomenon is an unspecific effect, the interaction of $LexA_{(1\text{-}87)408}$-EvgA with $LexA_{(1\text{-}87)WT}$-Fos was tested. It was expected that EvgA does not interact with Fos and therefore expression of the reporter gene would not be repressed. However, this combination also resulted in a slightly elevated repression compared to the samples of BglJ or GadE heterodimers. Therefore the values obtained with EvgA need to be normalized differently and are not likely to represent an actual repression.

Table 2: Summary of the FixJ/NarL-type transcription factor dimerization analyses obtained with the LexA two-hybrid system in *E. coli*.

	$LexA_{(1\text{-}87)WT}$[1]								
$LexA_{(1\text{-}87)408}$[1]	RcsB	BglJ	EvgA	GadE	RcsA	YjjQ	MatA	DctR	Fos
RcsB	8.6[2]	8.7[2]	7.6[3]	3.2[3]	8.1[4]	3.7[4]	8.7[3]	11.5[3]	–
BglJ	7.2		3.5	1.0	1.1	1.1[4]	0.9	0.9	–
EvgA	3.3	2.3		2.0	2.1	2.4	2.0	1.4	2.3
GadE	2.6	1.3	3.0		1.1	1.1	1.1	1.0	–
Jun	–	–	–	–	–	–	–	–	18.7[2]

[1] Interactions of hybrid proteins were investigated in *E. coli* strain S3440 (Δ*rcsB*) unless otherwise indicated. Fold repression values were generated using β-galactosidase assays.

[2] Interactions were shown previously in *E. coli* strain S3442 (Δ*rcsB* Δ(*yjjP-yjjQ-bglJ*)) (Venkatesh et al., 2010).

[3] Interactions were shown previously (laboratory data, unpublished).

[4] Interactions were shown previously in *E. coli* strain S3442 (laboratory data, unpublished).

So far, all heterodimer pairs occuring within the FixJ/NarL-type transcription factor family include the response regulator RcsB. Most of the other FixJ/NarL-type transcription factors such as CsgD, MalT, and EvgA are capable of homodimer formation (Table 1). The three best documented RcsB heterodimer combinations are RcsB/RcsA (Vianney et al., 2005, Francez-Charlot et al., 2003), RcsB/BglJ (Venkatesh et al., 2010, Stratmann et al., 2012), and RcsB/GadE (Castanié-Cornet et al., 2010) (see chapter 1.4). In addition, dimerization analysis using the LexA-based bacterial two-hybrid system implicated the putative formation of further RcsB heterodimer pairs (laboratory data, unpublished). This is the case for RcsB/EvgA, RcsB/MatA, and RcsB/DctR heterodimers (Figure 13 and Table 2).

In conclusion, all interactions tested using the LexA-based bacterial two-hybrid system did not yield further heterodimer pairs beyond those previously described. The project was thereupon focussed on the investigation of YjjQ on the basis of a microarray data analysis.

2.2 Analysis of microarray data regarding genes affected by YjjQ

To identify genes regulated by the FixJ/NarL-type transcription factor YjjQ in *E. coli* a microarray was performed (laboratory data, unpublished). For this microarray the *yjjP-yjjQ-bglJ* locus was deleted in *E. coli* strain K-12 and UPEC strain CFT073, resulting in the strains S3922 (BW30270 Δ(*yjjP-yjjQ-bglJ*)::KD3) and KEC394 (CFT073 Δ(*yjjP-yjjQ-bglJ*)::KD3), respectively. The two strains were transformed with the plasmid pKERV17 expressing YjjQ derived from *E. coli* K-12 under the control of an IPTG-inducible tac promoter (Table 13). pKERV17 contains the *yjjQ* gene fused to the epsilon Shine-Dalgarno sequence (εSD) derived from the leader of gene 10 in phage T7. This sequence constitutes a part of the RBS on the mRNA and greatly enhances translation efficiency (Olins *et al.*, 1988). For that reason the microarray strains presumably expressed very high protein levels of YjjQ. The empty vector (pKES169) was used as a control. After induction of YjjQ expression, RNA was isolated and analyzed with the Affymetrix GeneChip *E. coli* Genome 2.0 Array at the Cologne Center for Genomics (CCG), University of Cologne, Germany. The array included the *E. coli* strain K-12 (MG1655 laboratory strain), UPEC strain CFT073, and two EHEC strains that remained unprobed.

A summary of the original microarray data can be found in the appendix (see chapter 5.2). In *E. coli* K-12 Δ(*yjjP-yjjQ-bglJ*), about 200 genes were differentially regulated following YjjQ overexpression with a factor greater than +/- 2. In UPEC strain CFT073 Δ(*yjjP-yjjQ-bglJ*), YjjQ overexpression resulted as well in differential regulation of about 200 genes with a factor greater than +/- 2. In both bacterial strains the proportion of downregulated genes was much bigger than the one of the upregulated genes. This observation implies that YjjQ seems to act mainly as a repressor.

To determine the genes that are affected most by YjjQ a threshold was set at a fold change value of +/- 5. Within this range, 31 genes in K-12 and 44 genes in CFT073 were downregulated. In *E. coli* K-12 as well as CFT073 the repressed genes are organized in 20 loci. Twelve of these putative YjjQ target loci are present in both strains whereas the other eight loci seem to be repressed in a strain-specific manner. YjjQ also showed an activating effect on the expression of some genes. Three genes in *E. coli* K-12 and one gene in CFT073 genes were upregulated more than 5-fold.

Table 3 lists all 33 loci differentially regulated by YjjQ in *E. coli* K-12 and CFT073 with the respective fold change values within the threshold and, if known, a brief description of their gene products. The gene *cra* does not lie within the range but is included in the selection because it is a global regulator of many genes. Table 4 lists the 25 putative YjjQ target loci with known function including a simplified functional classification. A detailed description of the functions of the encoded proteins and the relationship between the different putative YjjQ targets is given in the appendix (see chapter 5.3).

According to Table 4, YjjQ is a putative negative regulator of flagellar motility, adhesion, ECM production, biofilm formation, acid stress, and energy metabolism. In the following experiments performed to elucidate these putative functions of YjjQ in *E. coli*, the focus was set on the *flhDC* locus and the *bcs* locus encoding the master regulator of flagellar motility and proteins mediating the synthesis of the exopolysaccharide cellulose, respectively.

Table 3: Putative target loci most strongly regulated by YjjQ in *E. coli* K-12 and UPEC strain CFT073 identified by microarray data analysis.

Target locus	Fold change[1]		Gene product function
	K-12	CFT073	
adiY adiC	-39.6	-29.3	Arginine-dependent acid resistance
cbpAM	-25.3	-8.0	DNA-binding co-chaperone system
cra	-3.4	-1.9	Catabolite repressor/activator
csrB	-11.3	-5.2	Pleiotropic sRNA (regulator of CsrA)
dctR	-5.1	-3.7	FixJ/NarL-type transcription factor
dcuB	-5.1	-1.9	C_4-dicarboxylate transporter
fimB	-2.7	-6.7	Type 1 fimbriae regulatory protein
flhDC	1.0[2]	-9.8	Master regulator of motility
frdABCD	-3.5	-5.9	Fumarate reductase enzyme complex (FRD)
gfc	-53.1[3]	not present	Group 4 capsule formation
iap	-5.8	-3.9	Alkaline phosphatase isozyme conversion
kpsMT kslAB	not present	-6.4	ABC-transporter group 2 capsule
mdtJI	-31.1	-13.7	Spermidine exporter
nanCMS	-11.5	1.1	Sialic acid (Neu5Ac) metabolism
ompC	-36.6	-17.2	Osmoporin
panD	-11.4	-10.4	L-Aspartate-α-decarboxylase
rcnB	-5.0	-5.2	Cobalt and nickel resistance
ucpA	-22.0	-8.7	Furan resistance (oxidoreductase?)
uspF	-6.4	-10.6	Universal stress protein
yfiRNB	-23.4	-7.8	Biofilm formation/motility control
yhjR bcsQABZC *bcsEFG*	-15.5 -9.0	-12.9 -7.9	Cellulose synthesis
zur	-5.9	1.0	Regulation of zinc uptake
c0411	not present	-8.4	Unknown function
c1618 c1617 ymgD	-3.2	-21.4	
ybhL	-26.8	-19.3	
ydgD	-6.7	-4.7	
yfdY	-5.3	-7.8	
ymiA yciX	-23.3	-10.4	
yqfB	-4.0	-5.1	
yrbL	-5.9	-2.9	
fruBKA	7.3	-1.1	Fructose metabolism
spy	3.5	6.0	ATP-independent periplasmic chaperone
zinT	7.6	2.3	Zinc- and cadmium-binding protein

[1] Indicated are the fold change values for individual genes or for the first gene in an operon.

[2] *E. coli* K-12 strain S3922 (BW30270 Δ(*yjjP-yjjQ-bglJ*)::KD3) is non-motile.

[3] The *gfc* operon is not expressed in *E. coli* K-12 due to an IS*1* insertion element (see chapter 5.3).

Table 4: Overview of the putative YjjQ target loci in *E. coli*.

Target locus[1]	Gene product function	Functional classification	Positive and negative regulation of the locus	Cellular systems affected
adiY adiC	Arginine-dependent acid resistance	AdiY: Transcriptional regulator AdiC: Membrane protein of the bacterial cell wall	General: H-NS TCSs: RcsCDB, EvgAS Else: ?	Acid resistance
cbpAM	DNA-binding co-chaperone system	Other	General: Lrp TCSs: ? Else: ?	Nucleoid structure, protein quality control
cra	Catabolite repressor/ activator	Transcriptional regulator	General: ? TCSs: ? Else: Polyamines	Energy metabolism, curli expression
csrB	Pleiotropic sRNA (regulator of CsrA)	sRNA	?	Biofilm formation, pathogenicity, c-di-GMP metabolism, motility, energy metabolism
dctR	FixJ/NarL-type transcription factor	Transcriptional regulator (indirect YjjQ target?)	General: H-NS, AdiY TCSs: RcsCDB, EvgAS Else: ?	Acid resistance, pathogenicity
dcuB	C_4-dicarboxylate transporter	Membrane protein of the bacterial cell wall	General: FNR, cAMP/CRP TCSs: DcuSR Else: ?	Energy metabolism
fimB	Type 1 fimbriae regulatory protein	Other	General: NanR, H-NS, Lrp, LrhA, IHF TCSs: EnvZ-OmpR, RcsCDB Else: ?	Adhesion, pathogenicity
flhDC	Master regulator of motility	Transcriptional regulator	General: H-NS, cAMP/CRP, LrhA, CsrA, IHF TCSs: EnvZ-OmpR, RcsCDB Else: sRNAs	Motility, pathogenicity, curli expression
frdABCD	Fumarate reductase enzyme complex (FRD)	Membrane protein of the bacterial cell wall	General: FNR TCSs: DcuSR Else: ?	Energy metabolism, motility
fruBKA	Fructose metabolism	FruA, FruB: Membrane proteins of the bacterial cell wall (indirect YjjQ target?)	General: Cra, cAMP/CRP TCS: ? Else: ?	Energy metabolism
gfc	Group 4 capsule formation	Membrane protein of the bacterial cell wall	?	Biofilm formation, pathogenicity
iap	Alkaline phosphatase isozyme conversion	Other	?	

Target locus[1]	Gene product function	Functional classification	Positive and negative regulation of the locus	Cellular systems affected
kpsMT	ABC-transporter group 2 capsule	Membrane protein of the bacterial cell wall	?	Biofilm formation, pathogenicity
mdtJI	Spermidine exporter	Membrane protein of the bacterial cell wall	?	Polyamine homeostasis, protein synthesis
nanCMS	Sialic acid (Neu5Ac) metabolism	Membrane protein of the bacterial cell wall	General: NanR, cAMP/CRP TCSs: EnvZ-OmpR, CpxAR Else: ?	Pathogenicity
ompC	Osmoporin	Membrane protein of the bacterial cell wall	General: H-NS, Lrp, IHF TCSs: EnvZ-OmpR, CpxAR Else: sRNAs	Nutrient uptake
panD	L-Aspartate-α-decarboxylase	Other	?	β-Alanine metabolism
rcnB	Cobalt and nickel resistance	Other	General: RcnR TCSs: ? Else: ?	Metal homeostasis
spy	ATP-independent periplasmic chaperone	Other	General: ? TCSs: CpxAR, RcsCDB Else: ?	Protein quality control, curli formation
ucpA	Furan resistance (oxidoreductase?)	Other (indirect YjjQ target?)	General: Cra, cAMP/CRP, IHF TCSs: ? Else: ?	Furan resistance
uspF	Universal stress protein	Other	?	Adhesion, motility
yfiRNB	Biofilm formation/ motility control	Membrane protein of the bacterial cell wall	?	Biofilm formation, pathogenicity, c-di-GMP metabolism
yhjR bcsQABZC bcsEFG	Cellulose synthesis	Membrane protein of the bacterial cell wall	?	Biofilm formation, adhesion
zinT	Zinc- and cadmium-binding protein	(indirect YjjQ target?)	General: Zur TCSs: ? Else: ?	Metal homeostasis, pathogenicity, curli expression
zur	Regulation of zinc uptake	Transcriptional regulator	?	Metal homeostasis, pathogenicity

[1] A detailed discussion of each locus listed here including references can be found in the appendix (see chapter 5.3).

2.3 Morphotype expression in *E. coli*

The *bcs* locus mediates cellulose synthesis in bacteria. The exopolysaccharide cellulose is a principal component of the ECM. The *yhjRbcsQbcsABZC* and *bcsEFG* operons were found to be substantially repressed by the transcription factor YjjQ in a microarray (see chapter 2.2). Putative repression of the *bcs* locus by YjjQ implies that this protein may affect ECM composition. This interference would probably be reflected in colony morphology. Wild-type Enterobacteriaceae co-expressing cellulose and curli exhibit a distinctive morphotype termed red, dry and rough (rdar) when grown on media supplemented with Congo Red (also called rugose or wrinkled) (Römling, 2005). The diazo dye Congo Red specifically stains amyloid fibers such as curli (Hammar et al., 1995). Furthermore, it is a pH indicator that changes its color from blue at pH 3 to red at pH 5. The rdar morphotype can be used as a scale for multicelluar behavior as there is an evident correlation between highly structured morphologies and the ability to produce an ECM. Mutants lacking components of the ECM produce attenuated colony morphologies with less elaborated architectures.

Regulation of red, dry and rough (rdar) morphotype expression has mainly been studied in *S. typhimurium* and natural *E. coli* isolates (Römling, 2005). Wild-type strains express both the *bcs* (cellulose) and the *csg* (curli) loci resulting in the rdar morphotype on LB Congo Red agar plates (Zogaj et al., 2001) (Table 5). In strains deficient for *bcsA*, the catalytic subunit of cellulose synthase, cellulose production is abolished. Biofilm formation is slightly reduced and the morphotype changes to brown, dry and rough (bdar). In strains deficient for *csgA*, the predominant structural component of curli, curli production is abrogated. Biofilm formation is severely inhibited and the morphotype changes to pink, dry and rough (pdar). In mutant strains lacking *csgD* encoding the ECM master regulator, the morphotype is called smooth and white (saw). The same applies for *bcsA csgA* double mutant strains (Table 5). Mutants of *csgD* as well as *bcsA csgA* double mutants lack all signs of biofilm formation such as auto-aggregation in liquid culture or adherence to glass (Zogaj et al., 2001).

Table 5: Characterization of *S. typhimurium* and natural *E. coli* isolates regarding multicellular behavior.

Genotype[1]	Curli expression	Cellulose production	Biofilm Formation[2]	Morphotype on Congo Red plates[2]
wild-type	+	+	+++	red, dry and rough (rdar)
Δ*bcsA*	+	-	++	brown, dry and rough (bdar)
Δ*csgA*	-	+	+	pink, dry and rough (pdar)
Δ*csgD*	-	-	-	smooth and white (saw)
Δ*bcsA* Δ*csgA*				

[1] *bcsA* encodes the catalytic subunit of cellulose synthase. *csgA* encodes the predominant structural component of curli. *csgD* encodes the ECM master regulator controling cellulose and curli synthesis.
[2] (Zogaj et al., 2001).

2.3.1 Phenotypic analysis of *E. coli* strains defective in curli and/or cellulose production

First I investigated the colony morphologies of the wild-type *E. coli* K-12 strain MG1655 (S3836) and of the wild-type UPEC strains CFT073 and 536 on LB Congo Red agar plates. This indicator medium was applied for every morphotype analysis (Römling, 2001). UPEC strain 536 exhibited the red, dry and rough (rdar) morphotype at 28°C (Figure 14) which made it a suitable strain to investigate the morphotypes of diverse mutants in more detail.

UPEC strain CFT073 displayed the smooth and white (saw) morphotype at 28°C and 37°C (Figure 14). This strain was previously shown to neither produce curli (as estimated by Congo Red binding) nor cellulose (as estimated by Calcofluor White binding) (Hancock *et al.*, 2007). When grown at 28°C, *E. coli* K-12 strain MG1655 (S3836) showed a colony morphology that did not resemble any morphotype described for *S. typhimurium* and natural *E. coli* isolates. The colonies were round and had an almost smooth appearance. They were overall brown-colored indicating that at least one ECM component might be expressed (Figure 14). Morphotype expression of CFT073 and of MG1655 was not further analyzed and the subsequent experiments were continued using UPEC strain 536.

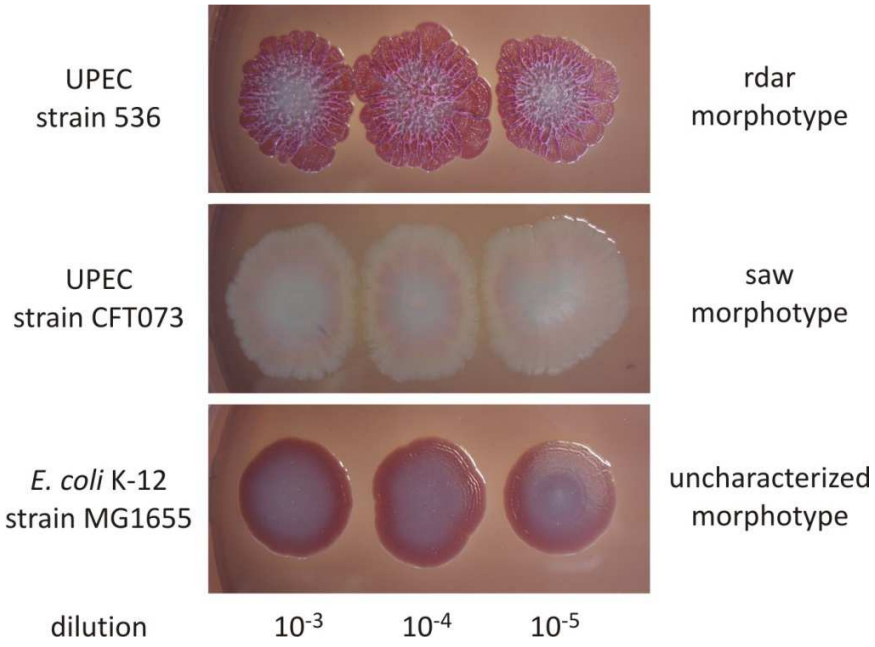


Figure 14: Morphotype analysis of wild-type *E. coli* strains.
Morphotype expression of wild-type *E. coli* K-12 strain MG1655 (S3836) and wild-type UPEC strains CFT073 and 536 was investigated on indicator plates. The rdar morphotype of 536 indicates co-expression of curli and cellulose. The saw morphotype of CFT073 indicates that neither curli nor cellulose is expressed. The colony morphology of MG1655 does not resemble any morphotype previously described. Small parts of single colonies were resuspended in water and diluted as indicated. 1 µl of each dilution was spotted on LB Congo Red agar plates. Morphotypes as observed after 7 days of growth at 28°C.

To recapitulate the morphotypes described for *S. typhimurium* and natural *E. coli* isolates in UPEC strain 536, *csgA*, *csgD* and/or *bcsA* deletion mutants were constructed by λ-Red mediated recombination (Datsenko & Wanner, 2000) (Figure 15). For this purpose the chromosomal sequences of the target genes or loci were replaced with a chloramphenicol resistance gene. For the construction of double mutant strains, the resistance cassette of a mutant deficient for the first gene was excised by Flp recombination (Cherepanov & Wackernagel, 1995). This chloramphenicol-sensitive strain was then transduced using EB49 phages (Battaglioli *et al.*, 2011) grown on a strain in which the second gene was already replaced with a chloramphenicol resistance gene.

For morphotype analysis of *csgA*, *csgD* and/or *bcsA* deletion mutants, the strains were grown on LB Congo Red agar plates for eight days at 28°C. The colony morphologies obtained in UPEC

536 mutants matched the morphotypes described for other natural *E. coli* isolates (Figure 15 and Table 6).

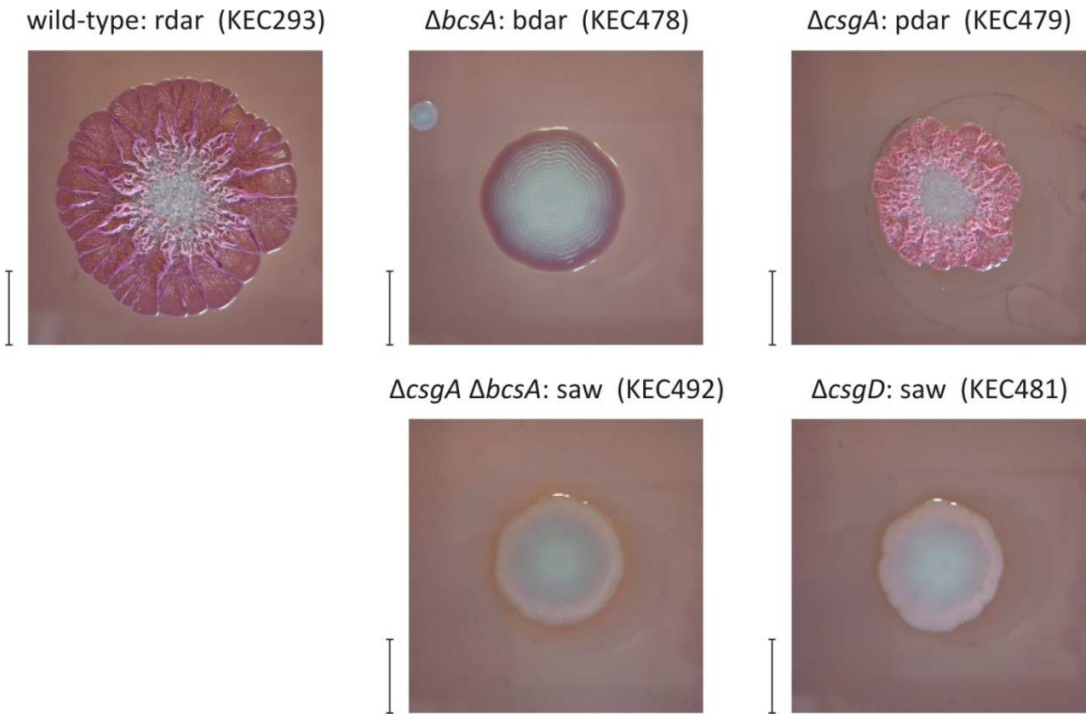


Figure 15: Deletions of the genes responsible for curli and cellulose production alter the morphotype of UPEC strain 536.
Morphotype expression of UPEC 536 wild-type and 536 deletion mutant strains was investigated on indicator plates. Wild-type 536 displays the rdar morphotype indicating co-expression of curli and cellulose. Deletion of *bcsA* encoding the catalytic subunit of cellulose synthase results in the bdar morphotype. Deletion of *csgA* encoding the predominant structural component of curli results in the pdar morphotype. Deletion of *csgD* encoding the ECM master regulator and deletion of a combination of *bcsA* and *csgA* results in the saw morphotype indicating that neither curli nor cellulose is expressed. Morphotypes as observed after 8 days of growth at 28°C on LB Congo Red agar plates. Scale bar: 1 cm.

Deletion of the cellulose gene *bcsA* caused the brown, dry and rough (bdar) morphotype. It was characterized by the formation of a round colony with a white center and a brown-colored rim (Figure 15). In addition, the formation of concentric bands on the colony surface was detected in this mutant. Deletion of the curli gene *csgA* caused the pink, dry and rough (pdar) morphotype reflected by a pink colony with an undulated rim. Irregular spokes emanating from the center of the colony resulted in the wrinkled appearance termed dry and rough. Strains lacking the ECM master regulator *csgD* or a combination of *csgA* and *bcsA* generated white

colonies of round shape representing the smooth and white (saw) morphotype. The colonies of all mutant strains tested were about half the size of the red, dry and rough (rdar) colonies occurring in the wild-type UPEC strain 536 (Figure 15).

2.3.2 Phenotypic analysis of *E. coli* strains defective in the *yjjQ* locus

The *bcs* locus is dedicated to the synthesis of the exopolysaccharide and principal ECM component cellulose in bacteria. Putative repression of this locus by the transcription factor YjjQ implies that this protein may affect ECM composition and therefore colony morphology. To analyze the effects of *bglJ*, *yjjQ-bglJ* and *yjjP-yjjQ-bglJ* mutations on the morphotype of UPEC strain 536, several deletion mutants were constructed by λ-Red mediated recombination (Datsenko & Wanner, 2000) (Figure 16).

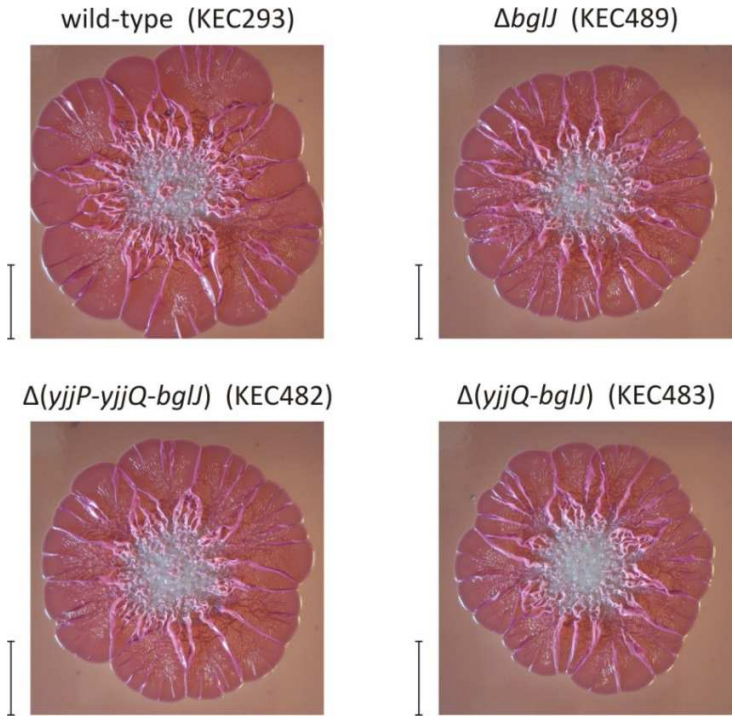


Figure 16: Deletions of the *yjjQ* locus genes do not alter the morphotype of UPEC strain 536.
Morphotype expression of UPEC 536 wild-type and deletion mutant strains was investigated on indicator plates. 536 Δ*bglJ*, Δ(*yjjP-yjjQ-bglJ*) and Δ(*yjjQ-bglJ*) strains exhibit the rdar morphotype. Morphotypes as observed after 8 days of growth at 28°C on LB Congo Red agar plates. Scale bar: 1 cm.

For morphotype analysis of the mutants deleted in the *yjjQ* locus, the strains were grown on LB Congo Red agar plates for eight days at 28°C. Deletions of *bglJ*, the *yjjQ-bglJ* operon and the *yjjP-yjjQ-bglJ* locus seem to have no effect on colony morphology since all strains displayed the red, dry and rough (rdar) morphotype (Figure 16). Color, structure as well as size of the colonies were indistinguishable from the wild-type indicating that deletion of *yjjQ* does not impinge upon ECM composition (Figure 16 and Table 6).

Table 6: Morphotype analysis of different UPEC 536 strains.

Strain name	Genotype[1]	Morphotype	Curli expression[2]	Cellulose production[2]
536	wild-type	rdar	+	+
KEC478/484	Δ*bcsA*::KD3/::FRT	bdar	+	-
KEC479/485	Δ*csgA*::KD3/::FRT	pdar	-	+
KEC481/488	Δ*csgD*::KD3/::FRT	saw	-	-
KEC491/496	Δ*bcsA*::FRT Δ*csgA*::KD3/::FRT	saw	-	-
KEC492+493/495	Δ*csgA*::FRT Δ*bcsA*::KD3/::FRT	saw	-	-
KEC482/487	Δ(*yjjP-yjjQ-bglJ*)::KD3/::FRT	rdar	+	+
KEC483/486	Δ(*yjjQ-bglJ*)::KD3/::FRT	rdar	+	+
KEC489/494	Δ*bglJ*::KD3/::FRT	rdar	+	+

[1] The first strain mentioned carries a chloramphenicol resistance gene derived from plasmid pKD3 and the second strain carries a FRT site due to excision of the resistance gene via Flp mediated recombination. Details on the construction of the strains are given in Table 12.

[2] *bcsA* encodes the catalytic subunit of cellulose synthase. *csgA* encodes the predominant structural component of curli. *csgD* encodes the ECM master regulator controling cellulose and curli synthesis.

2.3.3 Phenotypic analysis of *E. coli* strains expressing YjjQ

To further investigate the influence of the transcription factor YjjQ on the morphotype of UPEC strain 536, overexpression and complementation studies were performed. For this purpose the wild-type strain as well as Δ(*yjjP-yjjQ-bglJ*) and Δ(*yjjQ-bglJ*) strains were transformed with the respective plasmids expressing YjjQ under the control of an IPTG-inducible tac promoter (Figure 17). The *yjjQ* gene was either preceded by the native Shine-Dalgarno sequence (pKEKD30) or by the epsilon Shine-Dalgarno sequence (εSD) (pKEKD25) (Table 13). εSD is known to greatly enhance translation efficiency (Olins et al., 1988). For that reason pKEKD30 transformants

expressed moderate protein levels of YjjQ while pKEKD25 transformants presumably expressed very high YjjQ levels. The empty vector (pKESK22) was used as a control.

Plasmids pKEKD30 and pKEKD25 encode *yjjQ* originating from the UPEC strains 536 and CFT073, respectively, whereas *yjjQ* encoded by the plasmid pKERV17 used in the microarray originates from *E. coli* K-12. The gene products differ in a single amino acid at position 129 which is alanine in *E. coli* K-12 and serine in both UPEC strains. The concerned position is located in the middle of the protein upstream of the C-terminal DNA-binding domain. A comparison of the *yjjQ* sequence between *E. coli* K-12 and the UPEC strains 536 and CFT073 can be found in the appendix (see chapter 5.1).

For morphotype analysis the strains were grown on LB Congo Red agar plates for eight days at 28°C. 536 transformants of the vector control pKESK22 generated the red, dry and rough (rdar) morphotype independent of IPTG (Figure 17). IPTG-induced YjjQ expression in the wild-type from plasmid pKEKD30 yielding moderate protein levels reproducibly weakened the rdar morphotype compared to the vector control. The colonies were still red-colored but clearly attenuated regarding the dry and rough appearance. IPTG-induced YjjQ expression from pKEKD30 in the Δ(*yjjP-yjjQ-bglJ*) and Δ(*yjjQ-bglJ*) strains generated comparable results (Figure 17). The rdar morphotypes were weakened following complementation as in the wild-type background. Similar to the deletion mutant strains, the colonies of transformant strains treated with IPTG were smaller than the rdar colonies occurring in the control.

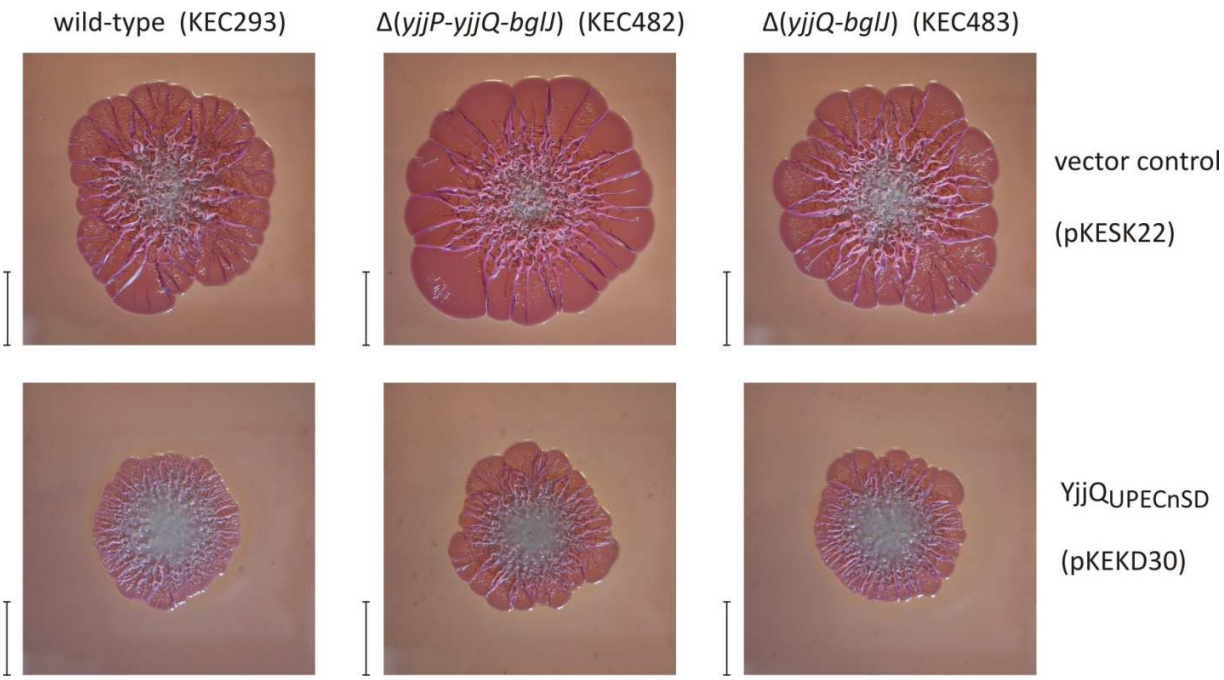


Figure 17: YjjQ expression alters the morphotype of UPEC strain 536.
Morphotype expression of UPEC 536 transformants was investigated on indicator plates. Overexpression of *yjjQ* in wild-type 536 leads to an attenuation of the rdar morphotype. Complementation of mutant 536 strains carrying *yjjQ* locus deletions leads to an attenuation of the rdar morphotype as well. Morphotypes as observed after 8 days of growth at 28°C on LB Congo Red agar plates supplemented with 0.2 mM IPTG and 25 µg/ml kanamycin. Scale bar: 1 cm.

Uninduced expression of YjjQ from pKEKD25 yielding high protein levels provoked colony morphologies that resembled the attenuated red, dry and rough (rdar) morphotype of IPTG-induced pKEKD30 transformants (Figure 18). Induction of YjjQ expression from pKEKD25 resulted in red-colored colonies of a round shape with a white rim and an almost smooth surface. The peculiar morphotypes of the pKEKD25 transformant can probably be attributed to the consequences of an excessive YjjQ protein level. The plasmid pKEKD25 contains *yjjQ* fused to εSD which means that the YjjQ concentration in this strain reached highly unphysiological levels that may be deleterious for cell metabolism.

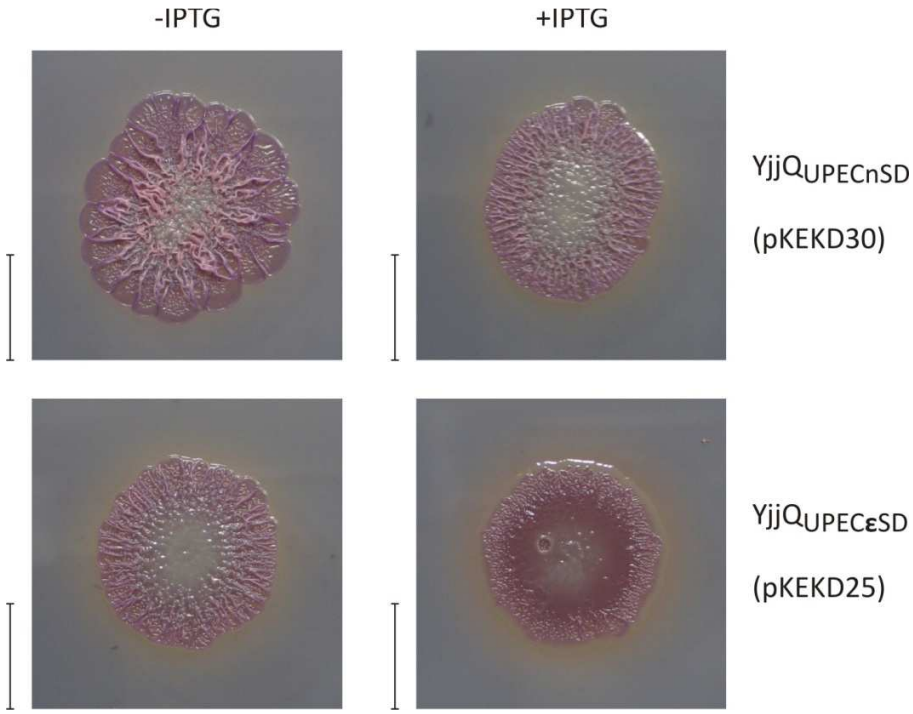


Figure 18: Morphotype analysis of UPEC 536 strains expressing different amounts of YjjQ.
Morphotype expression of UPEC 536 transformants was investigated on indicator plates. Basal level expression of *yjjQ* from plasmid pKEKD30 carrying the native Shine-Dalgarno sequence results in the rdar morphotype. IPTG-induced expression of *yjjQ* from pKEKD30 results in the attenuated rdar morphotype. Basal level expression of *yjjQ* from plasmid pKEKD25 carrying the epsilon Shine-Dalgarno sequence results in the attenuated rdar morphotype as well. IPTG-induced expression of *yjjQ* from pKEKD25 results in a strongly impaired morphotype. The blue color of the LB Congo Red agar plates may result from an acidic pH. Morphotypes as observed after 8 days of growth at 28°C on LB Congo Red agar plates +/- 0.2 mM IPTG and 25 µg/ml kanamycin. Scale bar: 1 cm.

In conclusion, these data suggest that YjjQ acts as a suppressor of multicellular behavior and possibly adhesion of Enterobacteriaceae. The mechanism by which this suppression is achieved is unknown at this point.

2.4 Biofilm formation of *E. coli* K-12 is impaired following YjjQ expression

The microarray performed in *E. coli* K-12 and UPEC strain CFT073 detected several putative target loci of the transcription factor YjjQ associated with multicellular behavior (Table 3). In addition to the *bcs* locus encoding proteins that mediate cellulose synthesis, expression of the *flhDC* locus encoding the master regulator of flagellar motility was downregulated as well. The exopolysaccharide cellulose is a principal component of the ECM and therefore represents a biofilm determinant (Zogaj et al., 2001, Solano et al., 2002). Motility is important especially during the early stages of biofilm formation (Pratt & Kolter, 1998) and for the development of the elaborated architecture of mature biofilms (Wood et al., 2006).

To investigate whether YjjQ has an impact on biofilm formation in *E. coli* K-12 strain BW30270 and UPEC strain 536, a biofilm assay was performed. This assay is based on the staining of adherent organic material to polystyrene (Merritt *et al.*, 2005). BW30270 is a K-12 derivative that is supposed to possess poor adhesive capacities because of low curli expression (see chapter 1.3.1). Wild-type cells were transformed with the respective plasmids expressing YjjQ under the control of an IPTG-inducible tac promoter. The *yjjQ* gene was either preceded by the native Shine-Dalgarno sequence (pKEKD30 and pKEKD31) or by the epsilon Shine-Dalgarno sequence (εSD) (pKERV17 and pKEKD25) (Table 13). εSD is known to greatly enhance translation efficiency (Olins et al., 1988). Plasmids pKEKD31 and pKERV17 encode *yjjQ* originating from *E. coli* K-12 whereas *yjjQ* encoded by the plasmids pKEKD30 and pKEKD25 originates from the UPEC strains 536 and CFT073, respectively. The empty vector (pKESK22) was used as a control.

The capacity of bacteria to form a biofilm can easily be assessed in a microtiter plate format (Merritt et al., 2005). The biofilm assay is carried out with samples in quadruplicates. Uninduced and IPTG-induced *E. coli* K-12 BW30270 as well as UPEC 536 transformants were grown in each of four wells of two 96-well microtiter plates in the presence of kanamycin. To rule out that components of the growth medium cause any unspecific staining, a LB control was included. The plates were incubated for 24 hours at 37°C and for 48 hours at 28°C, respectively. The wells were washed once and then stained with 0.1% crystal violet solution. After that the wells were washed twice and the plates were air-dried. Finally 30% acetic acid was added to each stained well to solubilize the dye.

Generally, the quadruplicates showed similar results with only few outliers. UPEC strain 536 hardly produced any biofilms at 28°C and 37°C as reflected by very weak staining (data not shown). Biofilm formation of *E. coli* K-12 strain BW30270 was faint at 37°C (data not shown) but strong at 28°C (Figure 19). Transformants of the vector control pKESK22 generated stable biofilms independent of IPTG. Basal level expression of YjjQ from pKEKD30 and pKEKD31 yielding moderate protein levels did not affect biofilm formation as the extent of the staining was indistinguishable from the control. Induction of YjjQ expression with 0.2 mM IPTG resulted in impaired biofilm formation (Figure 19 A). Surprisingly, induction with 1 mM IPTG had no impact compared to the uninduced samples (Figure 19 B). This implies that YjjQ efficiently represses biofilm formation on polystyrene at 28°C when expressed at physiological levels.

Basal level expression of YjjQ from pKEKD25 and pKERV17 yielding high protein levels led to a strongly reduced biofilm formation compared to the control. However, induction of YjjQ expression with 0.2 mM IPTG provoked partially increased biofilm formation (Figure 19 A) and induction with 1 mM IPTG caused slightly enhanced biofilm formation (Figure 19 B). The behavior of the pKEKD25 and pKERV17 transformant strains can probably be attributed to the consequences of excessive YjjQ protein levels described earlier.

The results obtained from the biofilm assay are consistent with those from the morphotype analysis. In UPEC strain 536, YjjQ seems to suppress multicellular behavior as its expression decreased the red, dry and rough (rdar) morphotype. Accordingly, adherence of *E. coli* K-12 to polystyrene is suppressed as well. However, it is not clear why UPEC strain 536 has a reduced capability of biofilm formation in the microtiter plate assay irrespective of the temperature.

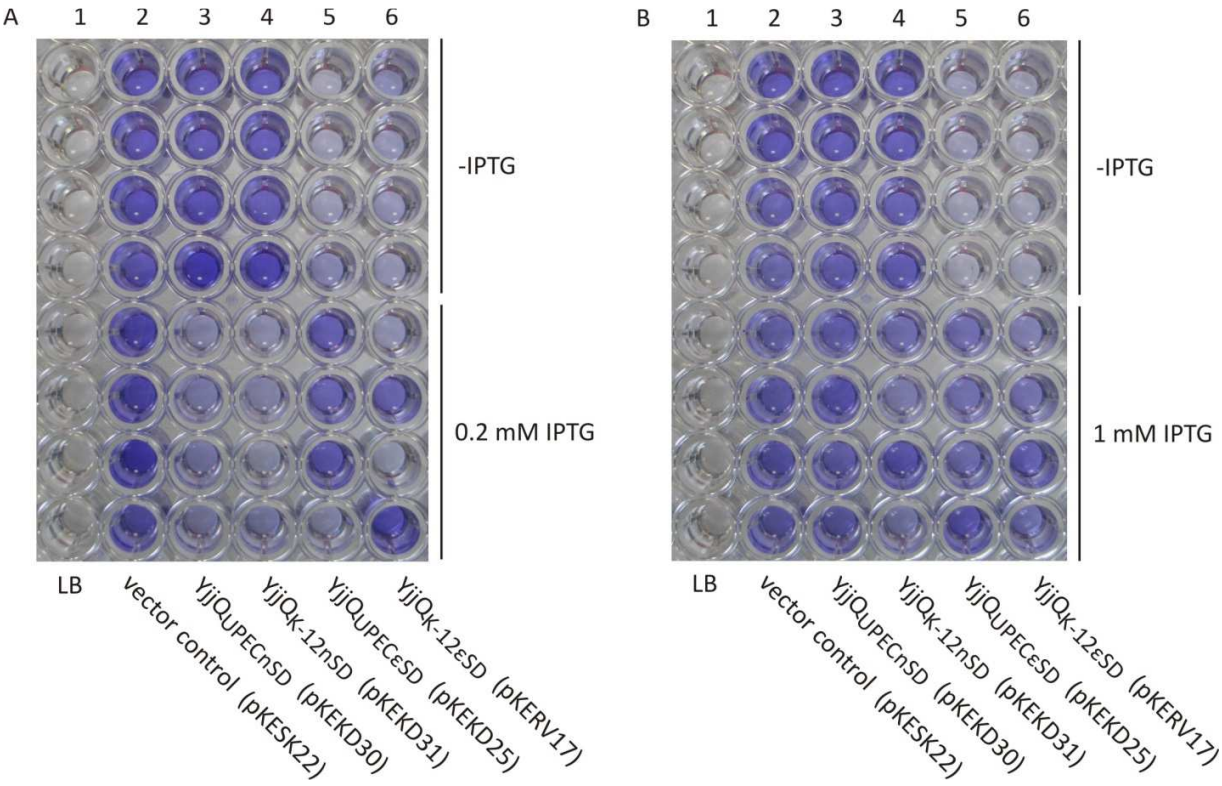


Figure 19: Biofilm formation of *E. coli* K-12 at 28°C is repressed by YjjQ.
The influence of YjjQ expression on the biofilm formation of *E. coli* K-12 strain BW30270 was investigated using the biofilm assay. Lane 1 shows the growth medium control and lane 2 shows the vector control. Lanes 3 to 6 show BW30270 transformants harboring plasmids expressing YjjQ under the control of an IPTG-inducible tac promoter. Transformants of the vector control pKESK22 generated stable biofilms in an IPTG-independent manner (panel A and B lane 2). Basal level expression of YjjQ from plasmids pKEKD30 and pKEKD31 carrying the native Shine-Dalgarno (nSD) sequence did not affect biofilm formation (panel A and B lanes 3 and 4). Induction of YjjQ expression from pKEKD30 and pKEKD31 with 0.2 mM IPTG results in impaired biofilm formation (panel A lanes 3 and 4). Induction of YjjQ expression from pKEKD30 and pKEKD31 with 1 mM IPTG had no impact on biofilm formation (panel B lanes 3 and 4). Basal level expression of YjjQ from plasmids pKEKD25 and pKERV17 carrying the epsilon Shine-Dalgarno sequence leads (εSD) to strongly reduced biofilm formation (panel A and B lanes 5 and 6). Induction of YjjQ expression from pKEKD25 and pKERV17 with 0.2 mM IPTG results in partially enhanced biofilm formation (panel A lanes 5 and 6). Induction of YjjQ expression from pKEKD25 and pKERV17 with 1 mM IPTG causes slightly increased biofilm formation (panel B lanes 5 and 6). Strains were grown in LB medium +/- IPTG and 25 µg/ml kanamycin at 28°C for 48 hours. Samples were analyzed in quadruplicates.

2.5 Motility of *E. coli* K-12 and UPEC strain 536 is repressed by YjjQ

One of the loci substantially repressed by the transcription factor YjjQ in the UPEC strain CFT073 derivative KEC394 is the *flhDC* locus encoding the master regulator of flagellar motility (Table 3). In *E. coli* K-12 strain S3922 (BW30270 Δ(*yjjP-yjjQ-bglJ*)::KD3) no regulation of *flhDC* was detected probably because this strain is defective in motility as was realized when the microarray was already performed. To investigate whether YjjQ has an impact on motility in *E. coli* K-12 strain T1241 (BW30270 ilvG+) and UPEC strain 536, a motility assay was performed. Strain T1241 is known to be motile. The motility capacity of UPEC strain 536 was unknown and needed to be tested. For that reason the 536 wild-type strain was included in the motility assay. Wild-type cells were transformed with the respective plasmids expressing YjjQ under the control of an IPTG-inducible tac promoter. The *yjjQ* gene was either preceded by the native Shine-Dalgarno sequence (pKEKD30 and pKEKD31) or by the epsilon Shine-Dalgarno sequence (εSD) (pKERV17 and pKEKD25) (Table 13). Plasmids pKEKD31 and pKERV17 encode *yjjQ* originating from *E. coli* K-12 whereas *yjjQ* encoded by the plasmids pKEKD30 and pKEKD25 originates from the UPEC strains 536 and CFT073, respectively. The empty vector (pKESK22) was used as a control.

For assessment of motility the strains were grown on LB soft agar plates for five to six hours at 37°C. The influence of YjjQ on motility was inferred from comparison of the swarm radii between the control and strains expressing YjjQ. In *E. coli* K-12 strain T1241, transformants of the vector control pKESK22 exhibited motility independent of IPTG (Figure 20 A). Basal level expression of YjjQ from plasmids pKEKD30 and pKEKD31 yielding moderate protein levels led to a motility pattern that was indistinguishable from the control (+/- 1 mm). Induction of YjjQ expression with 0.2 mM IPTG eliminated motility (Figure 20 B and C). Basal level expression of YjjQ from plasmids pKEKD25 and pKERV17 yielding high protein levels led to a loss of motility compared to the control. As expected, induction of YjjQ expression with 0.2 mM IPTG made no difference since motility was already absent in the uninduced strains (Figure 20 D and E).

Wild-type UPEC strain 536 turned out to be motile as well (Figure 21 A). Transformants of the vector control pKESK22 exhibited motility independent of IPTG (Figure 21 B). Basal level expression of YjjQ from pKEKD30 and pKEKD31 led to a motility pattern that was

indistinguishable from the control. Induction of YjjQ expression with 0.2 mM IPTG strongly suppressed motility (Figure 21 C and D). Basal level expression of YjjQ from pKEKD25 and pKERV17 led to a decrease of motility. Induction of YjjQ expression with 0.2 mM IPTG eliminated motility (Figure 21 E and F).

These data suggest that YjjQ in deed negatively regulates motility of *E. coli* via repression of the flagellar *flhDC* locus. The results indicate that this locus, previously identified as a putative YjjQ target in the microarray of UPEC strain CFT073, is an actual YjjQ target.

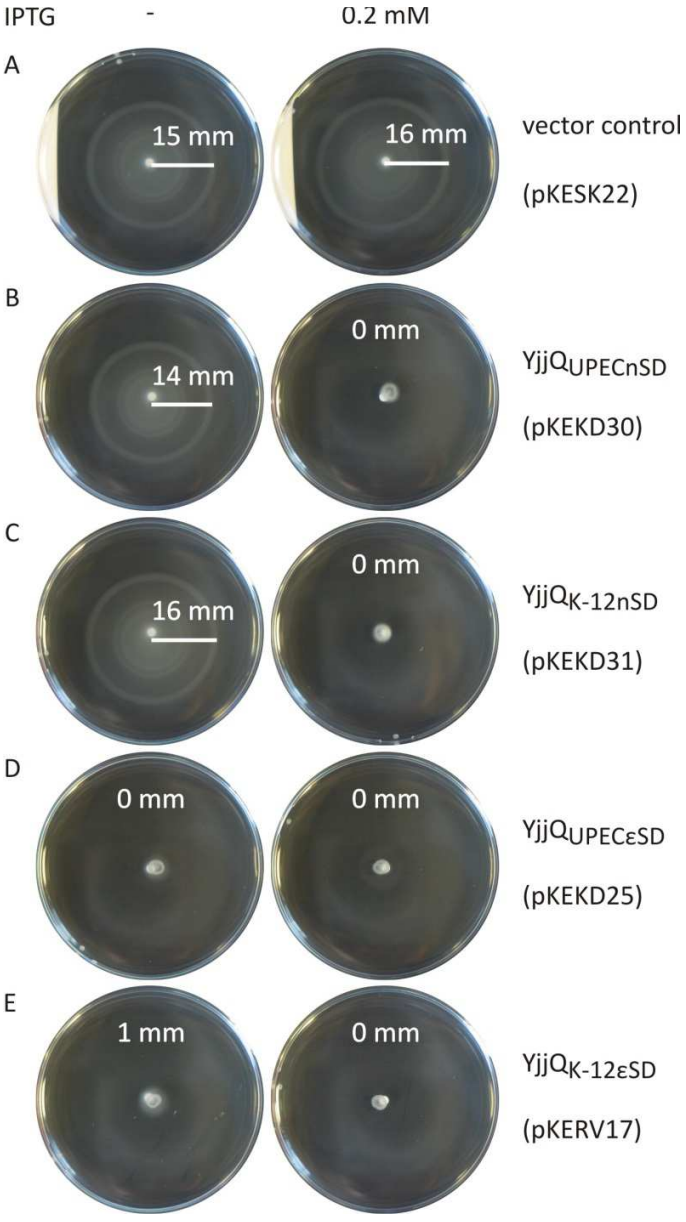


Figure 20: Motility of *E. coli* K-12 is repressed by YjjQ.
The influence of YjjQ expression on the motility of *E. coli* K-12 strain T1241 was investigated using the motility assay. Panel A shows the vector control. Panels B to E show transformants harboring plasmids expressing YjjQ under the control of an IPTG-inducible tac promoter. Transformants of the vector control pKESK22 exhibited motility in an IPTG-independent manner (panel A). Basal level expression of YjjQ from plasmids pKEKD30 and pKEKD31 carrying the native Shine-Dalgarno sequence (nSD) did not affect motility (left panel B and C). IPTG-induced YjjQ expression from pKEKD30 and pKEKD31 eliminated motility (right panel B and C). Basal level expression as well as IPTG-induced YjjQ expression from plasmids pKEKD25 and pKERV17 carrying the epsilon Shine-Dalgarno sequence (εSD) leads to a loss of motility (panels D and E). Strains were grown on LB soft agar plates +/- IPTG and 25 µg/ml kanamycin at 37°C for five hours.

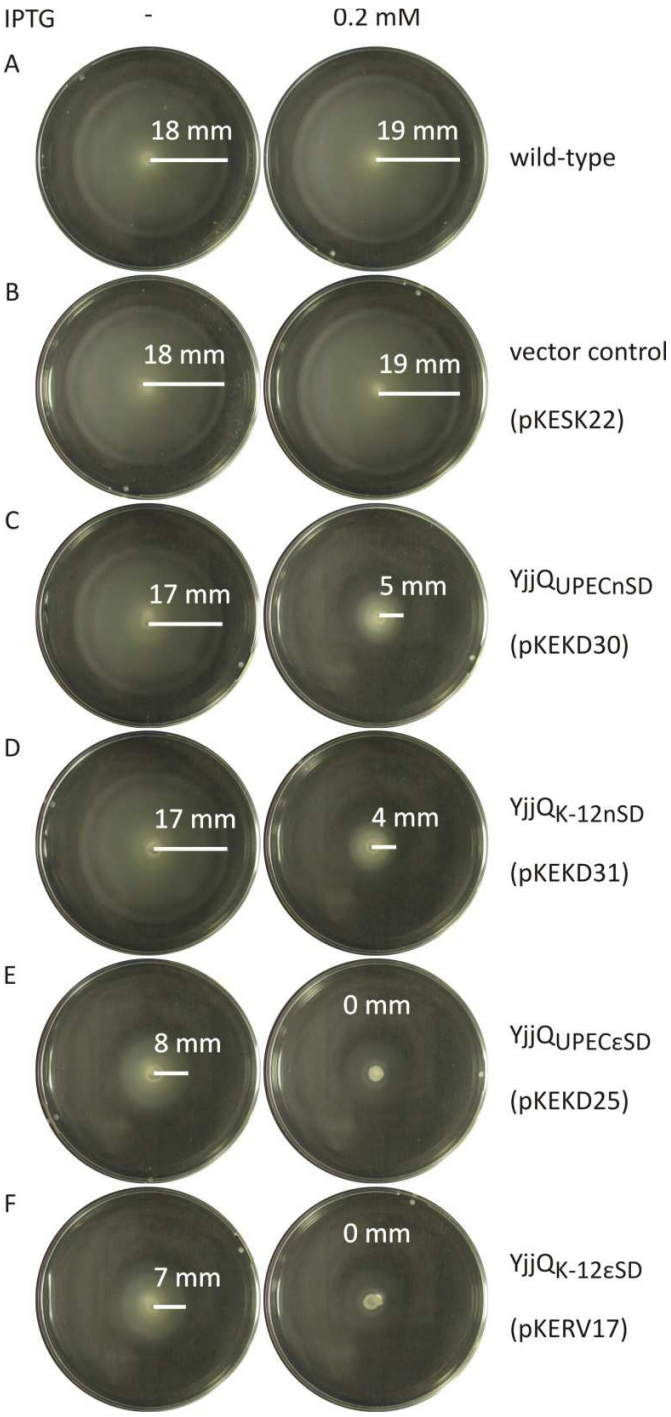


Figure 21: Motility of UPEC strain 536 is repressed by YjjQ.
The influence of YjjQ expression on the motility of UPEC strain 536 was investigated using the motility assay. Panel A shows the wild-type and panel B shows the vector control. Panels C to F show transformants harboring plasmids expressing YjjQ under the control of an IPTG-inducible tac promoter. Wild-type 536 as well as transformants of the vector control pKESK22 exhibited motility in an IPTG-independent manner (panels A and B). Basal level expression of YjjQ from plasmids pKEKD30 and pKEKD31 carrying the native Shine-Dalgarno sequence (nSD) did not affect motility (left panel C and D). IPTG-induced YjjQ expression from pKEKD30 and pKEKD31 with 0.2 mM IPTG strongly suppressed motility (right panel C and D). Basal level expression of YjjQ from plasmids pKEKD25 and pKERV17 carrying the epsilon Shine-Dalgarno sequence (εSD) leads to a decrease of motility (left panel E and F). Induction of YjjQ expression from pKEKD25 and pKERV17 with 0.2 mM IPTG eliminated motility (right panel E and F). Strains were grown on LB soft agar plates +/- IPTG and +/- 25 µg/ml kanamycin at 37°C for six hours.

2.6 Investigation of UPEC adhesion with the help of *in vitro* model systems

The microarray performed in *E. coli* K-12 and UPEC strain CFT073 detected several putative target loci of the transcription factor YjjQ associated with adhesion (Table 3). These loci include the *bcs* locus encoding proteins that mediate the synthesis of cellulose which is a principal component of the ECM and therefore represents a biofilm determinant. Adhesion capacities of bacteria to eukaryotic cells can be assessed by the use of adhesion assays. These assays are *in vitro* cell culture-based infection models. To examine the influence of the major ECM components curli and cellulose as well as of YjjQ on the adhesion capacity of UPEC to potential host cells, an adhesion assay was established. Using this assay, infections of urothelial 5637 cells with wild-type and mutant UPEC strains 536 and CFT073 were performed.

The effects of deletions of *csgA* encoding the predominant structural component of curli, *csgD* encoding the ECM master regulator controling cellulose and curli synthesis, and *bcsA* encoding the catalytic subunit of cellulose synthase have been studied before (Saldaña et al., 2009). These authors reported that single mutations in *csgA*, *csgD* or *bcsA* in EHEC O157:H7 and EPEC O127:H6 had no dramatic impact on cell adherence. However, double *csgA bcsA* mutants were observed to be less adherent than the single mutants or wild-type strains to human colonic HT-29 epithelial cells or to bovine colon tissue *in vitro*. Overexpression of CsgD in a *csgD* mutant, but not in the *csgA* or *bscA* single mutants, was shown to increase adherence to HT-29 cells in the EPEC and EHEC strains tested. Based on their findings they reasoned that simultaneous overexpression of curli and cellulose enhances bacterial adherence to eukaryotic cells (Saldaña et al., 2009).

2.6.1 Adhesion assay

The classical adhesion assay is a frequently used *in vitro* model system to determine adhesion capacities of bacteria to eukaryotic cells (Elsinghorst, 1994, Eto *et al.*, 2007). In collaboration with D. Gürlebeck (AG Wieler, FU Berlin) I have accomplished an adhesion assay with UPEC strain CFT073 on the canine kidney cell line MDCK. CFT073 Δ(*yjjP-yjjQ-bglJ*)::KD3 (KEC394) was transformed with plasmids either expressing *yjjQ* preceded by the epsilon Shine-Dalgarno sequence (εSD) or *bglJ*. The empty vector (pKESK22) was used as a control. The three transformant strains as well as the parent strain were plated on LB agar plates supplemented with kanamycin. On the assay day, single colonies of all four strains were used to inoculate LB medium. These cultures were grown to an OD_{600} of 2 to 3 in the presence of 1 mM IPTG and 25 µg/ml kanamycin. 1 ml of each culture was pelleted by centrifugation and resuspended in 1 ml cell culture medium. The cell count of the MDCK cells was determined and the OD_{600} of the CFT073 suspensions was measured. The inoculum was calculated to adjust the desired MOI value of 1. From the respective preparatory cultures, the inoculums were diluted in cell culture medium and vortexed briefly. The medium was aspirated from the cell culture plate and the inoculum was added. The plate was centrifuged to synchronize the infection and then incubated for one hour. The inoculum was aspirated and the samples were washed three times with PBS. For cell lysis Triton X100 in PBS was added to the plate. After a short incubation the lysates were harvested, serial dilutions were made and the two highest dilutions were plated in duplicates on LB agar plates. On the next day the colonies were counted and the evaluation of the obtained values as a graphical representation was done. CFT073 complemented with YjjQ showed a 6.7-fold decrease in adhesion compared to the wild-type whereas complementation with BglJ only had a marginal influence (data not shown). This experiment was only performed once so that the data were not reproduced.

As stated before, YjjQ may affect adhesion through an inhibiton of ECM production via repression of the *bcs* locus mediating cellulose exopolysaccharide synthesis. Curli and cellulose have been demonstrated to act synergistically to promote adherence of EHEC and EPEC (Saldaña et al., 2009). In order to find out if this oberservation is transferable to other *E. coli* species, I intended to reproduce the data in a UPEC model system. To investigate if defective

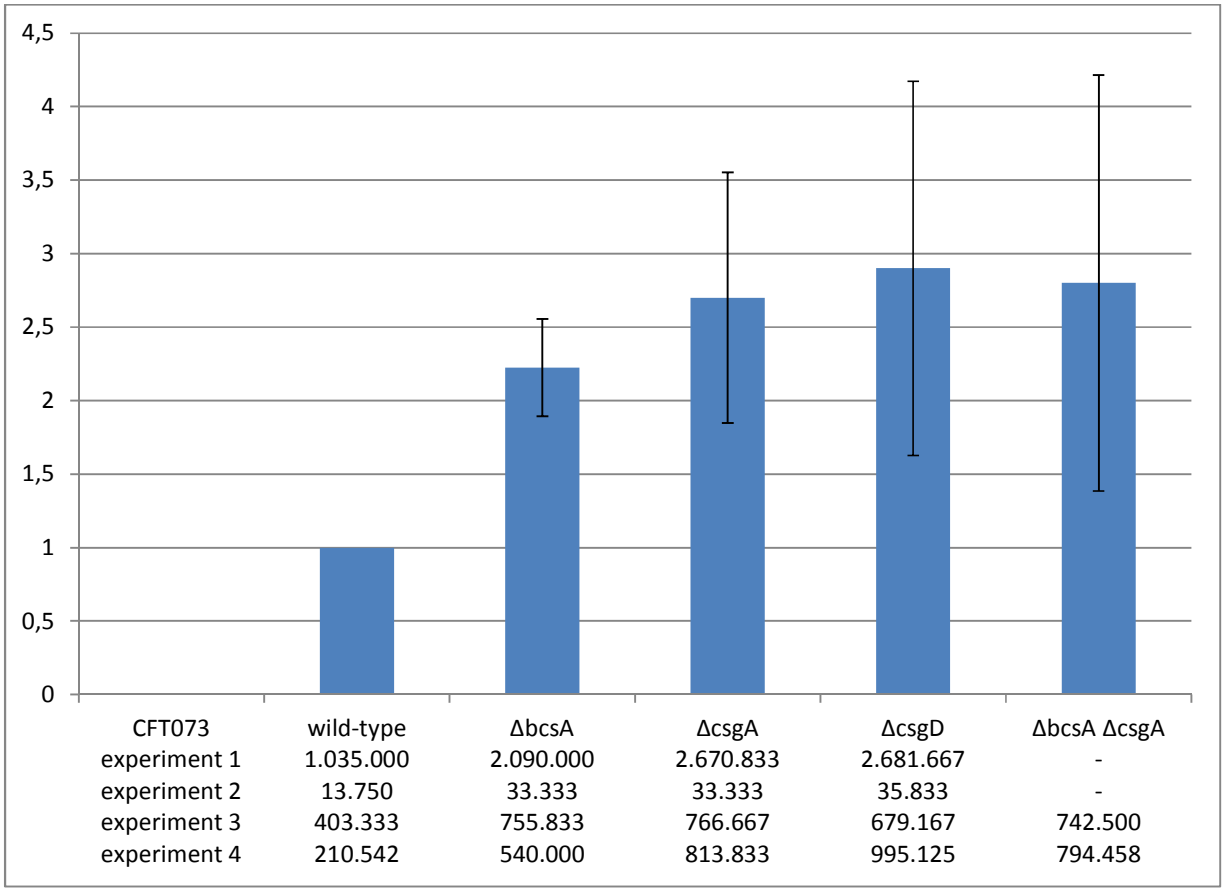
curli and/or cellulose production has consequences on the adhesion capacity of UPEC strain CFT073, the deletion mutant strains Δ*bcsA* (KEC466/467), Δ*csgA* (KEC468/469) Δ*csgD* (KEC470/471), and Δ*bcsA* Δ*csgA* (KEC472) were constructed by λ-Red mediated recombination (Datsenko & Wanner, 2000) (Table 11). Eukaryotic cells were infected with the CFT073 wild-type and deletion mutant strains using a modified protocol. The MDCK cell line was replaced with the epithelial carcinoma cell line 5637 derived from the human urinary bladder (CLS cell lines service, Eppelheim). Instead of using colonies from plates for the inoculation of the preparatory cultures, overnight cultures were grown in cell culture medium. Accordingly, the preparatory cultures were grown in cell culture medium as well. This procedure omits the centrifugation and resuspension step. Furthermore, the preparatory cultures were grown to exponential phase with an OD_{600} of 0.5 instead of using stationary growth phase cells.

In each experiment, wild-type UPEC CFT073 (KEC375) adhered the least efficiently compared to all deletion mutants, which is in contrast to the previous observations (Saldaña et al., 2009). Adherence of the deletion mutant strains to 5637 cells is increased between 2.2-fold and 2.9-fold in average compared to the wild-type (Figure 22). The fact that CFT073 neither produces curli nor cellulose (Hancock et al., 2007) was unknown by the time the adhesion assays were performed. For that reason the data concerning the mutants with defective curli and/or cellulose production are obsolete and are not discussed in more detail.

In an attempt to find out if YjjQ negatively affects adhesion, I infected urothelial 5637 cells with UPEC strain CFT073. Infection was carried out with wild-type CFT073 as well as with a transformant strain harboring the plasmid pKEKD25 expressing YjjQ derived from UPEC under the control of the IPTG-inducible tac promoter. The *yjjQ* gene was preceded by the epsilon Shine-Dalgarno sequence (εSD) which is known to greatly enhance translation efficiency (Olins et al., 1988). Therefore expression of YjjQ from pKEKD25 yields high protein levels. The empty vector (pKES169) was used as a control. Adherence of the transformant expressing YjjQ was increased 3.8-fold in average compared to the wild-type (Figure 23). Transformants of the vector control pKES169 showed even a 12.7-fold increase in adhesion to 5637 cells. This is in contradiction to the preliminary result obtained from the adhesion assay performed in Berlin where CFT073 complemented with YjjQ showed a 6.7-fold decrease in adhesion compared to

the wild-type. The adhesion assay system I used could not support the observation previously made regarding the negative role of YjjQ on adhesion. However, the experimental conditions, the genetic background of the parent strain as well as the plasmid encoding *yjjQ* were different. Despite these differences that may account for the opposite results, the classical adhesion assay was abandoned because it entails many other disadvantages as summarized below.

After having performed the adhesion assay several times it emerged that the reproducibility cannot be guaranteed as illustrated by the high standard deviations. Since the number of colonies differs substantially between the assays, only the samples of one single experiment can be correlated reliably to each other. The strong discrepancies between the colony numbers may result from the necessity to use high dilutions of the samples to generate single colonies. For titer determination of adherent bacteria, the colony numbers are multiplied with the dilution factor which results in a wide error margin. Moreover, it is not possible to distinguish between bacteria that adhere to eukaryotic cells and bacteria that adhere unspecifically to the surface. To be able to give a definite statement on the function of YjjQ in adhesion, its mechanisms of action need to be studied in much greater detail. For that reason I switched to a microscopy-based approach.



CFT073	wild-type	ΔbcsA	ΔcsgA	ΔcsgD	ΔbcsA ΔcsgA
experiment 1	1.035.000	2.090.000	2.670.833	2.681.667	-
experiment 2	13.750	33.333	33.333	35.833	-
experiment 3	403.333	755.833	766.667	679.167	742.500
experiment 4	210.542	540.000	813.833	995.125	794.458

Figure 22: Adhesion of wild-type and deletion mutant UPEC CFT073 strains to 5637 cells.
Bacterial adhesion was analyzed with the adhesion assay. Urothelial 5637 cells were infected with UPEC CFT073 strains with different genetic backgrounds. CFT073 wild-type was used as reference for the calculation of changes in adhesion. The wild-type strain adheres the least efficiently of all strains tested. Deletion of *bcsA* encoding the catalytic subunit of cellulose synthase results in a 2.2-fold increase in adhesion. Deletion of *csgA* encoding the predominant structural component of curli results in a 2.7-fold increase in adhesion. Deletion of *csgD* encoding the ECM master regulator results in a 2.9-fold increase in adhesion. Deletion of a combination of *bcsA* and *csgA* results in a 2.8-fold increase in adhesion. The bars represent the average fold-changes calculated from four (WT, Δ*bcsA*, Δ*csgA*, Δ*csgD*) or two (Δ*bcsA* Δ*csgA*) experiments, respectively. On the X-axis the individual values per experiment are indicated.

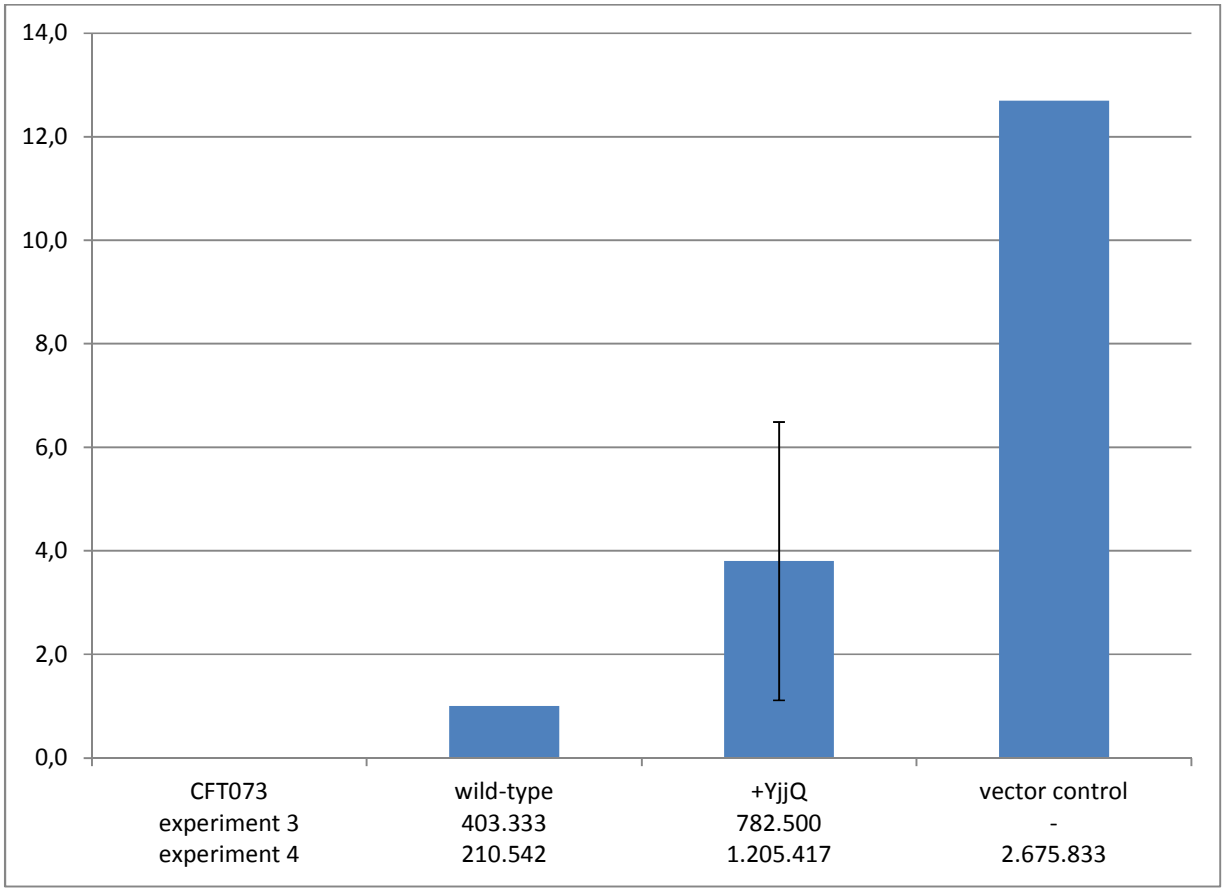


Figure 23: Influence of YjjQ on the adhesion of UPEC CFT073 to 5637 cells.
Bacterial adhesion was analyzed with the adhesion assay. Urothelial 5637 cells were infected with UPEC CFT073 strains with different genetic backgrounds. CFT073 wild-type was used as reference for the calculation of changes in adhesion. The wild-type strain adheres the least efficiently of all strains tested. Overexpression of YjjQ preceded by the epsilon Shine-Dalgarno sequence results in a 3.8-fold increase of adhesion. Transformants of the vector control showed a 12.7-fold increase in adhesion. The bars represent the average fold-changes calculated from two (WT, +YjjQ) or one (vector control) experiment, respectively. On the X-axis the individual values per experiment are indicated. The following plasmids were used: $YjjQ_{UPEC\varepsilon SD}$ (pKEKD25) and vector control (pKES169).

2.6.2 Microscopic visualization of *E. coli* adhesion

An extended variation of the classical adhesion assay is the microscopy-based adhesion assay. This approach allows the microscopic visualization of fluorescently labelled bacteria and eukaryotic cells. Both methods serve the purpose to determine adhesion capacities of UPEC to potential host cells, but the microscopic approach further allows monitoring of immediate interactions of the bacteria with the eukaryotic cells. Another crucial advantage is the possibility to distinguish bacteria that adhere to the surface from bacteria that are associated to eukaryotic cells. For that reason the degree of adhesion can be calculated as adherent bacterial cell per eukaryotic cell compared to adherent bacteria in total. For the microscopy-based adhesion assay, UPEC strain 536 was chosen as strain to be used because 536 expresses both curli and cellulose as illustrated by the rdar morphotype (Figure 14).

In order to gain a fluorescent UPEC 536 parent strain, a genetic construct was cloned comprising the constitutive P_L promoter, the epsilon Shine-Dalgarno sequence (εSD), a green fluorescent protein (GFP) reporter, and a selectable resistance gene. The final fluorescence reporter construct is depicted in Figure 24.

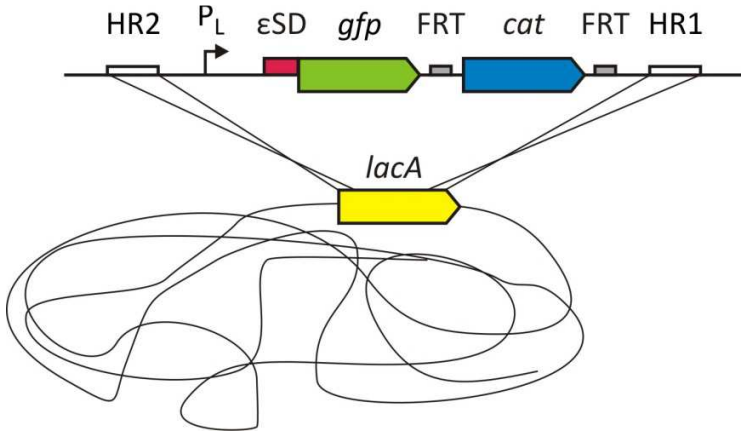


Figure 24: Fluorescence reporter construct in UPEC 536 strains used for the microscopy-based adhesion assay.
Chromosomal *lacA* was replaced with the depicted promoter-reporter fusion containing the constitutive P_L promoter from phage λ, the epsilon Shine-Dalgarno sequence εSD, the reporter gene *gfp*, and the chloramphenicol resistance gene *cat* which is flanked by FRT sites. The promoter-reporter fusion was introduced into the chromosome of wild-type 536 by λ-Red mediated recombination using the *lacA* homology regions HR2 and HR1.

The P_L promoter was amplified from phage λ DNA and was subsequently cloned in the presence of plasmid pFDX334 encoding cI-857, a thermolabile mutant allele of the λcI repressor (George *et al.*, 1987). This protein represses P_L at 28°C, a condition that was needed for cloning of the otherwise constantly active promoter. The GFP reporter variant *gfp-mut1* (Cormack *et al.*, 1996) preceded by εSD originated from plasmid pKEIB16 (Table 13). The chloramphenicol resistance gene (*cat*) was flanked by Flp recombinase target (FRT) sites which allow excision of *cat* via Flp-mediated recombination. The P_L promoter and the chloramphenicol resistance gene were encompassed by regions homologous to upstream and downstream sequences of the chromosomal target gene *lacA* (Figure 24). These *lacA* homology regions termed HR1 and HR2 were generated by annealing of phosphorylated complementary oligonucleotides. The reporter cassette was introduced into the 536 chromosome by λ-Red mediated recombination according to Datsenko and Wanner thereby generating strain KEC490 (536 Δ*lacA*::P_L*gfp*-KD3) (Datsenko & Wanner, 2000).

To examine the influence of the major ECM components curli and cellulose as well as of YjjQ on the adhesion capacity of UPEC to potential host cells, several fluorescent deletion mutant strains were constructed. For the construction of these strains the UPEC 536 mutant collection generated for the morphotype analysis was used. The deletion mutant strains with excised resistance cassettes (KEC484-488, KEC494-496) were transduced with phage EB49 (Battaglioli et al., 2011) from phages grown on the fluorescent UPEC 536 parent strain Δ*lacA*::P_L*gfp*-KD3 (KEC490) (Table 12). By selecting chloramphenicol-resistant clones, fluorescent 536 mutant strains (KEC497-504) harboring deletions of the genes responsible for curli and/or cellulose production as well as of genes belonging to the *yjjQ* locus were generated (Table 12).

UPEC 536 Δ*lacA*::P_L*gfp*-KD3 (KEC490) was used to establish the staining and the experimental conditions for the microscopy-based adhesion assay (Figure 25). Urothelial 5637 cells were grown to 60-80% confluence so that the bacteria could in principal adhere to an abiotic surface represented by the glass cover slip as well as a biotic surface represented by the eukaryotic cells. The infection procedure is the same as the one of the classical adhesion assay and includes a centrifugation step. After infection, 5637 cells were stained with 2.5 µg/ml CellMask Orange in cell culture medium for 1 min. Samples were fixed with 1 ml 4% para-formaldehyde

in PBS for 15 min at 37°C and then mounted with Fluoroshield including DAPI. Figure 25 demonstrates that under the same experimental conditions, UPEC 536 appears to localize to the glass surface as well as to associate with eukaryotic cells.

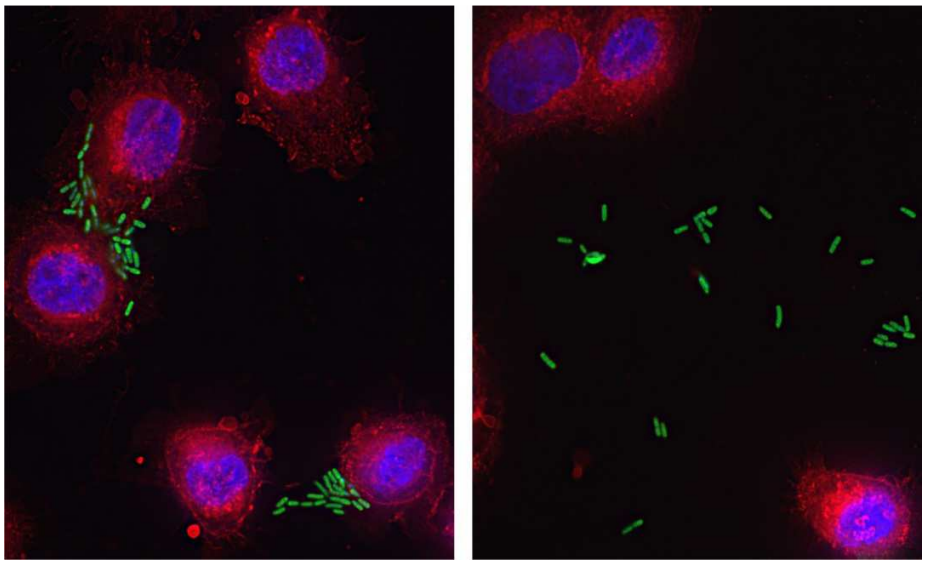


Figure 25: Image of fixed human urinary bladder carcinoma 5637 cells infected with the UPEC 536 GFP reporter strain KEC490.
Microscopy samples were treated with CellMask Orange staining the plasma membrane of urothelial 5637 cells, and afterwards fixed with 4% para-formaldehyde in PBS. Samples were mounted with Fluoroshield containing DAPI staining nucleic acids of the eukaryotic nucleus and of the bacterial chromosome. The image was taken with the widefield DeltaVision RT microscope (Applied Precision, Inc.) and processed with the softWoRx 5.5 image analysis software.

Infections of urothelial 5637 cells with reporter strains with defective curli and/or cellulose production were analyzed by microscopy and compared to UPEC 536 Δ*lacA*::P_L*gfp*-KD3 (KEC490). Two incubation times with defined MOI values were used for all assays. Samples infected with a MOI of 30 were incubated for 30 min while samples infected with a MOI of 2 were incubated for two hours. The fluorescent *E. coli* K-12 strain T66 (P_{lac}::*gfp-mut1* Δ*lacZYA*-kan) was intended to serve as a negative control because *E. coli* K-12 laboratory strains are supposed to produce only low amounts of adhesive structures. The overall number of T66 cells was indeed reduced compared to KEC490 (Table 7 and Figure 26). But the fact that T66 cells could be detected at all suggests that the bacteria, irrespective of the strain, do not actively adhere but instead may be forced onto the surface by centrifugation. This assumption is

underlined by the fact that if the centrifugation is omitted, almost no bacteria can be detected (data not shown). In addition, only very low amounts of bacteria could be detected in general compared to the applied MOI (Table 7). As already observed with the classical adhesion assay, the standard deviations were again very high.

To investigate the influence of YjjQ expression on bacterial adhesion, UPEC 536 Δ*lacA*::P_L*gfp*-KD3 (KEC490) was transformed with the plasmid pKEKD30 expressing YjjQ under the control of an IPTG-inducible tac promoter. The *yjjQ* gene was preceded by the native Shine-Dalgarno sequence and therefore expression of YjjQ from pKEKD30 yields moderate protein levels. The empty vector (pKESK22) was used as a control. For both infection conditions, expression of YjjQ from pKEKD30 resulted in bacterial cell numbers that were very similar to those found in the wild-type strain (Table 8 and Figure 27). Cell numbers of transformants of the vector control pKESK22 were elevated compared to the wild-type and the YjjQ transformants. The adhesion capacity of a UPEC 536 reporter strain harboring a deletion of the *yjjP-yjjQ-bglJ* locus was also assessed (536 Δ(*yjjP-yjjQ-bglJ*)::FRT Δ*lacA*::$P_L$gfp-KD3). Surprisingly, the cell numbers of this deletion mutant strain were even more increased than those of the vector control transformants (Table 8 and Figure 27).

In summary, neither the classical adhesion assay nor the microscopy-based adhesion assay were sufficient to analyze UPEC adhesion to urothelial cells adequately. These results illustrate that conclusions deduced from adhesion assays need to be evaluated very critical. All data published based on adhesion assays should be supported by other methods that guarantee higher reliability and reproducibility.

Table 7: Adhesion of wild-type UPEC 536 and *E. coli* K-12 GFP reporter strains to 5637 cells.

UPEC 536 strain/ K-12 strain[1]	Triplicate No	5637 nuclei[2]	co-localized bacteria[2]	glas-attached bacteria[2]	total bact./ 1000 cells	co-loc. bact./ 1000 cells	Average total bact./ 1000 cells	Stdev total	Stdev% total	Average co-loc. bact./ 1000 cells	Stdev co-loc.	Stdev% co-loc.
KEC490 Δ*lacA*::$P_L$gfp-KD3 2h MOI=2	1	483	85	69	319	176	172 (9% of MOI)	115	67%	105 (5% of MOI)	56	54%
	2	424	42	25	158	99						
	3	1207	47	0	39	39						
KEC490 Δ*lacA*::$P_L$gfp-KD3 30min MOI=30	1	794	152	30	229	191	430 (1% of MOI)	146	34%	221 (1% of MOI)	30	13%
	2	582	122	163	490	210						
	3	531	139	165	573	262						
T66 P_{lac}::*gfp-mut1* Δ*lacZYA*-kan 2h MOI=2	1	1191	50	0	42	42	46 (2% of MOI)	24	52%	46 (2% of MOI)	24	52%
	2	458	35	0	76	76						
	3	1063	20	0	19	19						
T66 P_{lac}::*gfp-mut1* Δ*lacZYA*-kan 30min MOI=30	1	509	17	46	124	33	209 (1% of MOI)	64	31%	67 (0% of MOI)	25	37%
	2	514	47	96	278	91						
	3	853	65	127	225	76						

[1] Bacterial cultures were grown overnight to stationary phase in cell culture medium.

[2] The raw data was obtained with the help of the Volocity 6.1.1 image analysis software either by automatic (5367 nuclei) or maunal (bacteria) counting.

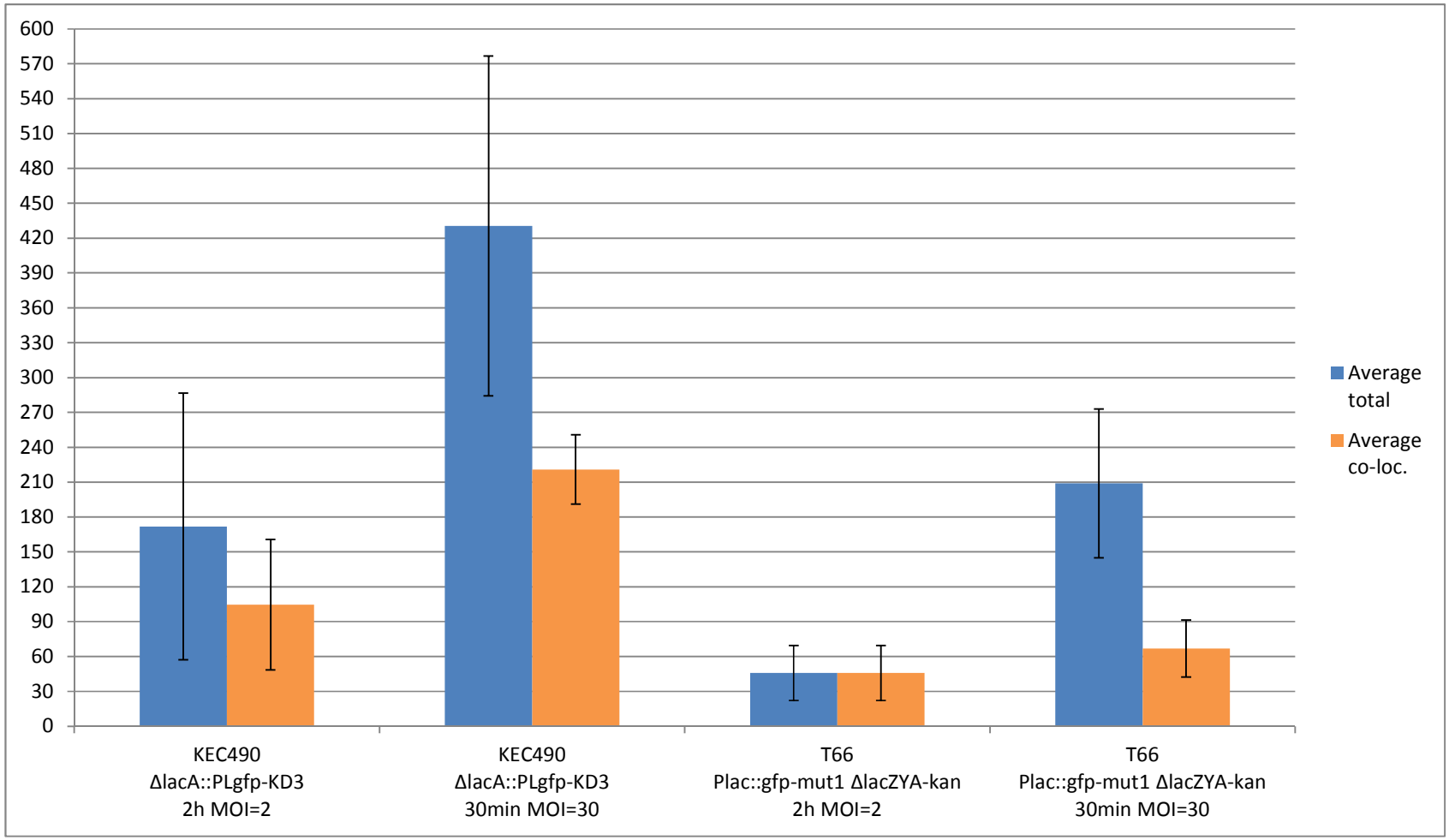


Figure 26: Adhesion of UPEC 536 as well as of *E. coli* K-12 GFP reporter strains to 5637 cells.
Urothelial 5637 cells were infected with the UPEC 536 strain KEC490 (Δ*lacA*::P_L*gfp*-KD3) and the *E. coli* K-12 strain T66 (P_{lac}::*gfp-mut1* Δ*lacZYA*-kan). Bacterial adhesion was analyzed with the microscopy-based adhesion assay. Although T66 shows reduced adhesion compared to KEC490 it is able to adhere unspecifically to the surface as well as co-localized with 5637 cells. The chart depicts the calculated averages of total adherent bacteria per 1000 eukaryotic cells and of co-localized adherent bacteria per 1000 eukaryotic cells including the standard deviations. Infection was carried out using the conditions indicated on the X-axis.

Table 8: Influence of YjjQ on the adhesion of UPEC 536 GFP reporter strains to 5637 cells.

UPEC 536 strain[1]	Triplicate No	5637 nuclei[2]	co-localized UPEC[2]	glas-attached UPEC[2]	total UPEC/ 1000 cells	co-loc. UPEC/ 1000 cells	Average total UPEC/ 1000 cells	Stdev total	Stdev% total	Average co-loc. UPEC/ 1000 cells	Stdev co-loc.	Stdev% co-loc.
KEC490 Δ*lacA*::$P_L$gfp-KD3 2h MOI=2	1	740	136	2	186	184	96 (5% of MOI)	72	75%	93 (5% of MOI)	71	76%
	2	770	9	0	12	12						
	3	614	52	3	90	85						
KEC490 Δ*lacA*::$P_L$gfp-KD3 30min MOI=30	1	617	84	21	170	136	73 (0% of MOI)	69	95%	60 (0% of MOI)	54	89%
	2	763	22	0	29	29						
	3	697	11	2	19	16						
KEC490 + YjjQ 2h MOI=2	1	859	43	0	50	50	96 (5% of MOI)	96	100%	80 (4% of MOI)	74	92%
	2	452	82	22	230	181						
	3	551	5	0	9	9						
KEC490 + YjjQ 30min MOI=30	1	833	44	4	58	53	71 (0% of MOI)	39	55%	60 (0% of MOI)	31	51%
	2	564	57	13	124	101						
	3	802	22	3	31	27						
KEC490 + vector control 2h MOI=2	1	561	57	11	121	102	143 (7% of MOI)	105	73%	117 (6% of MOI)	82	70%
	2	413	93	23	281	225						
	3	823	21	1	27	26						
KEC490 + vector control 30min MOI=30	1	581	76	125	346	131	303 (1% of MOI)	156	52%	132 (0% of MOI)	36	28%
	2	618	54	4	94	87						
	3	481	85	141	470	177						
KEC500 Δ(*yjjP-yjjQ-bglJ*) 2h MOI=2	1	545	83	1	154	152	237 (12% of MOI)	64	27%	233 (12% of MOI)	60	26%
	2	903	224	0	248	248						
	3	666	198	8	309	297						
KEC500 Δ(*yjjP-yjjQ-bglJ*) 30min MOI=30	1	734	54	82	185	74	527 (2% of MOI)	415	79%	230 (1% of MOI)	149	65%
	2	524	97	52	284	185						
	3	480	207	326	1110	431						

[1] Bacterial cultures were grown overnight to stationary phase (KEC490 Δ*lacA*::$P_L$gfp-KD3 and KEC500) or to exponential phase in the presence of 1 mM IPTG (KEC490 + YjjQ and KEC490 + vector control) in cell culture medium.

[2] The raw data was obtained with the help of the Volocity 6.1.1 image analysis software either by automatic (5367 nuclei) or maunal (UPEC) counting.

The following plasmids were used: $YjjQ_{UPECnSD}$ (pKEKD30) and vector control (pKESK22).

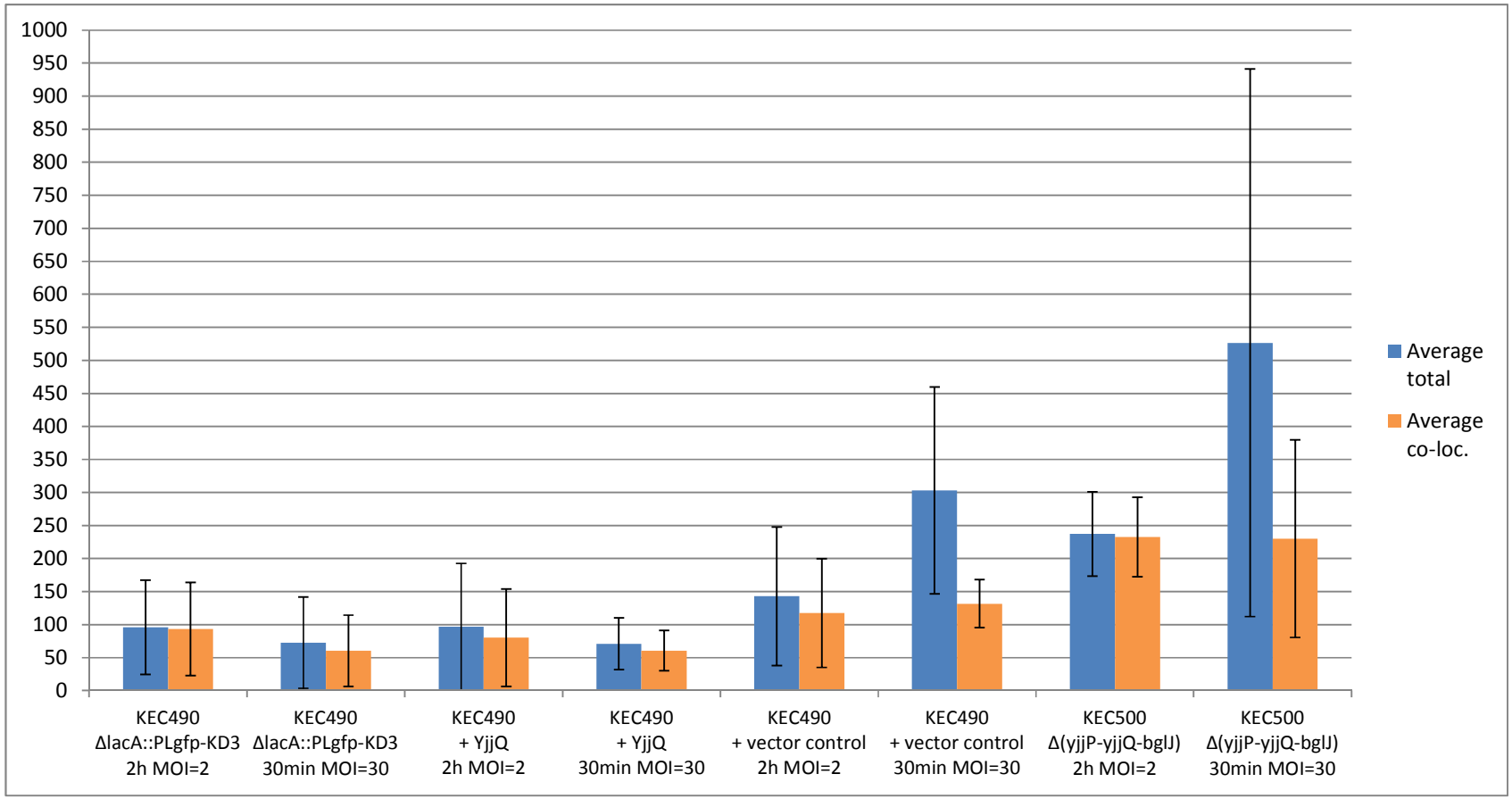


Figure 27: Influence of YjjQ on the adhesion of UPEC 536 GFP reporter strains to 5637 cells.
Urothelial 5637 cells were infected with four UPEC 536 strains with different genetic backgrounds. Bacterial adhesion was analyzed with the microscopy-based adhesion assay. 536 transformants expressing YjjQ adhered to the same extent than the wild-type 536 strain. Transformants of the vector control showed enhanced adhesion compared to wild-type and YjjQ transformants. The 536 strain KEC500 carrying a deletion of the *yjjQ* locus showed increased adhesion compared to the vector control. The chart depicts the calculated averages of total adherent bacteria per 1000 eukaryotic cells and of co-localized adherent bacteria per 1000 eukaryotic cells including the standard deviations. The following plasmids were used: $YjjQ_{UPECnSD}$ (pKEKD30) and vector control (pKESK22). Infection was carried out using the conditions indicated on the X-axis.

3. Discussion

In this study novel functions of the previously uncharacterized FixJ/NarL-type transcription factor YjjQ were identified. Microarray data analysis revealed that YjjQ constitutes a transcriptional repressor of multiple loci in *E. coli* K-12 and UPEC. Furthermore, it was shown that YjjQ represses motility in *E. coli* K-12 and UPEC. This effect can be attributed to YjjQ-mediated repession of the master regulator of flagellar motility. Moreover, YjjQ was demonstrated to repress morphotype expression in UPEC and biofilm formation in *E. coli* K-12. These findings provide the basis for further experiments in order to elucidate the role of YjjQ in bacterial physiology, behavior, and pathogenicity.

3.1 YjjQ is a pleiotropic repressor in *E. coli*

To identify genes regulated by the transcription factor YjjQ in *E. coli* K-12 and UPEC strain CFT073 a microarray was performed. One of the most important findings inferred from the microarray data analysis is the fact that YjjQ seems to act mainly as a transcriptional repressor. The putative YjjQ target genes are involved in various unrelated pathways, but several loci can be linked to the regulation of biofilm formation. For instance, some of the affected gene products play roles in adhesion processes (*bcs, fimB*), capsule synthesis (*gfc, kpsMT*), and motility control (*flhDC*).

Five genes encoding transcriptional regulators are negatively affected by YjjQ: *adiY* involved in acid resistance, the catabolite repressor/activator protein *cra* involved in energy metabolism, the FixJ/NarL-type transcription factor *dctR* involved in acid resistance and pathogenicity, the pleiotropic motility regulator *flhDC*, and *zur* involved in zinc uptake (only in K-12) (see chapter 2.2). Strikingly, a large part of the genes downregulated by YjjQ encode membrane proteins that are located in the outer membrane (OM) and/or the inner membrane (IM) of the bacterial cell wall. This is the case for the 10 loci listed in Table 9. This suggests that YjjQ may have a substantial influence on the composition of mainly the inner membrane and thereby on cell wall integrity.

Table 9: Putative YjjQ target loci encoding membrane proteins of the bacterial cell wall in *E. coli* K-12 and UPEC strain CFT073.

Target locus[1]	Gene product function	Cell wall location
adiC	arginine-agmatine antiporter	IM
bcsA	cellulose synthase	IM
mdtJI	spermidine exporter	IM
ompC	osmoporin	OM
yfiN yfiB	DGC and lipoprotein	IM and OM
dcuB	fumarate-succinate antiporter	IM
gfc	group 4 capsule formation	OM/IM
nanC	Neu5Ac-specific porin	OM
frdABCD	fumarate reductase	IM
kpsMT	capsular polysaccharide ABC-transporter	IM

[1] Fold repression values are given in Table 3.

There are two possible mechanisms by which YjjQ-mediated repression of target genes could be achieved. On the one hand YjjQ could bind directly to sequences in the target gene promoters via its HTH DNA-binding domain. This direct interaction could in turn interfere with the binding of activating transcription factors or with RNA polymerase itself thereby preventing transcription. On the other hand YjjQ could act indirectly by repressing the expression of transcriptional regulators. This mechanism seems likely for the putative target gene *ucpA* since YjjQ downregulates expression of Cra, a known transcriptional activator of this gene, in *E. coli* K-12 (Sirko *et al.*, 1997) (Table 4). Moreover, Cra is a negative regulator of the *fruBKA* operon (Ramseier *et al.*, 1993). Repression of Cra by YjjQ is therefore reflected in the elevated expression of *fruBKA* (Table 4). Interestingly, a Cra-binding site was determined on the *yjjP* open reading frame by a SELEX approach (systematic evolution of ligands by exponential enrichment). Thus, YjjQ and BglJ are putative members of the transcription factor network organized in the Cra regulon (Shimada *et al.*, 2011c).

Other examples for a putative indirect influence of gene expresion by YjjQ are the 7.6-fold upregulation of *zinT* and the 5.1-fold downregulation of *dctR* in *E. coli* K-12. The *zinT* gene is repressed by Zur, a transcription factor known to regulate zinc uptake in *E. coli* (Patzer & Hantke, 2000, Graham *et al.*, 2009). Elevated expression of *zinT* can be explained by the

downregulation of its repressor Zur by YjjQ in *E. coli* K-12 (Table 4). Repression of *dctR* may also be an indirect effect through YjjQ-mediated repression of its transcriptional activator AdiY (Krin *et al.*, 2010a) (Table 3). Beyond the modes of YjjQ action described here, other mechanisms such as protein-protein interactions that are not detected by the microarray method may occur as well. Furthermore, additional YjjQ target loci that are not represented on the Affymetrix GeneChip *E. coli* Genome 2.0 Array probably exist.

Regarding the microarray data analysis, one should keep in mind that this method does not provide a quantification of immediate transcriptional activity on individual promoters. Many indirect effects resulting from a complex interplay of small regulatory RNAs (Yus *et al.*, 2012, Lalaouna *et al.*, 2013), RNA-degrading enzymes (Arraiano *et al.*, 2010), RNA-binding proteins such as Hfq and CsrA (Nogueira & Springer, 2000), and other effectors like polyamines (Igarashi & Kashiwagi, 2010) affect mRNA synthesis, stability, and processing. The extent of the actions of these additional positive or negative regulators on the transcriptome remain unconsidered. In addition, the test strain used in the microarray was transformed with the plasmid pKERV17 containing *yjjQ* fused to the epsilon Shine-Dalgarno sequence (εSD), a genetic element known to greatly enhance translation efficiency. But this also means that the YjjQ concentration in the test strain reached highly unphysiological levels that may be deleterious for cell metabolism including transcription events. For these reasons more direct approaches need to be applied in the future to validate the microarray data.

3.2 Morphotype analysis

The red, dry and rough (rdar) morphotype exhibited by Enterobacteriaceae reflects co-expression of cellulose and curli and represents an indicator for community behavior. Phenotypic analysis of *yjjQ*-deficient UPEC 536 strains revealed that deletion of this gene has no impact on colony morphology. The wild-type strain as well as the Δ*bglJ*, Δ(*yjjQ-bglJ*), and Δ(*yjjP-yjjQ-bglJ*) mutant strains exhibited indistinguishable rdar morphotypes on LB Congo Red agar plates after eight days of growth at 28°C. These results were anticipated since YjjQ is virtually not expressed *in vivo* due to H-NS-mediated repression (Stratmann et al., 2008). In contrast, overexpression and complementation with YjjQ from the plasmid pKEKD30 yielding moderate

protein levels leads to a pronounced suppression of the rdar morphotype (Figure 17). The colonies were smaller and less wrinkly compared to the control. The phenotype obtained from the overexpression and complementation experiments suggests that YjjQ acts as a suppressor of multicellular behavior. The mechanism by which this suppression is achieved is not understood. YjjQ-mediated repression of the *bcs* locus dedicated to cellulose synthesis could not be confirmed by promoter-*lacZ* fusions (see below, chapter 3.6). The effects of YjjQ on morphotype expression may be implemented via a different route than regulation of the *bcs* promoters.

As stated before, microarray data analysis revealed YjjQ-mediated downregulation of Cra in *E. coli* K-12. This global transcriptional regulator was shown to be involved in biofilm formation through the regulation of curli synthesis (see chapter 1.3.1). *E. coli* K-12 strains carrying a *cra* mutation form colorless colonies on indicator plates due to abolished Congo Red-binding (Reshamwala & Noronha, 2011). Accordingly, the *csgDEFG* operon was found to be 2.8-fold downregulated by YjjQ in K-12. In addition, another locus associated with ECM production, the *ydaM* gene encoding a diguanylate cyclase responsible for c-di-GMP synthesis, was 2.5-fold downregulated by YjjQ in K-12. No differential regulation of these loci was detected in UPEC strain CFT073. This is consistent with the observation that CFT073 neither produces curli nor cellulose (Hancock et al., 2007). YjjQ-mediated regulation of the two loci in UPEC strain 536 is not known. One possibility to examine gene repression by YjjQ in 536 would be the performance of qRT-PCR on the putative target genes identified in the microarray. Nevertheless, the results from the morphotype analysis imply a role for YjjQ in biofilm formation and possibly adhesion.

3.3 Biofilm assay

In Enterobacteriaceae, curli as well as cellulose production are essential factors required for biofilm formation and adhesion (Zogaj et al., 2001, Saldaña et al., 2009). The capacity of *E. coli* laboratory strains to form stable biofilms is controversial. *E. coli* strains lacking curli expression and/or cellulose synthesis may possess a yet uncharacterized collection of surface adhesins

with different binding specificities that are expressed only under certain environmental conditions (Beloin *et al.*, 2008).

Curli expression is maximally induced in media without salt within a temperature range of 26°C to 30°C (Olsén et al., 1989, Bokranz et al., 2005). This observation was confirmed by the biofilm assay in which *E. coli* K-12 strain BW30270 showed much stronger biofilm formation at 28°C than at 37°C (see chapter 2.4). Plasmidic expression of YjjQ induced by 0.2 mM IPTG considerably repressed adherence of *E. coli* K-12 strain BW30270 to polystyrene at 28°C (Figure 19). The effect of YjjQ on biofilm formation of UPEC strain 536 could not be examined with this method because this strain hardly produced any biofilms in microtiter plates at 28°C and 37°C.

Most wild-type *E. coli* K-12 derivatives produce curli levels that are insufficient for proper adherence even though the curli genes are fully functional (Vidal et al., 1998). The concerned strains have lost the environmental control of the *csg* operons e.g. by temperature or osmolarity due to mutations in regulatory genes like *rpoS* or in the *csgD* promoter itself. Therefore they depend on an inactivating mutation of a repressor like H-NS (Olsén et al., 1993) or an activating mutation of an activator like the osmoregulatory response regulator OmpR to induce curli synthesis (Gualdi et al., 2007, Vidal et al., 1998). In contrast, most natural *E. coli* isolates have an inherent capacity to form biofilms on different biotic and abiotic surfaces. UPEC strain 536 belongs to this group as illustrated by the formation of the red, dry and rough (rdar) morphotype on LB Congo Red agar plates. However, 536 did not produce a biofilm on polystyrene irrespective of the growth temperature. A possible explanation could be the use of LB as growth medium instead of a medium with a reduced salt content. If this fact is in deed the cause of the observed biofilm defect, it would emphasize the strong dependence of biofilm formation on the growth conditions of different bacterial strains.

Besides curli and cellulose, biofilm formation is largely influenced by other substances as well. Additional ECM constituents are e.g. colanic acid, extracellular DNA (eDNA), and poly-β-1,6-N-acetyl-D-glucosamine (PGA) (Branda et al., 2005, Flemming & Wingender, 2010). The structure of the exopolysaccharide colanic acid resembles the group 1 capsule of *E. coli* (Whitfield, 2006). Its biosynthesis locus underlies transcriptional control by the RcsCDB TCS (Majdalani & Gottesman, 2005). Colanic acid is essential for *E. coli* K-12 biofilm formation (Danese et al.,

2000). High-level expression of colanic acid interferes with adhesion of UPEC to various surfaces (Hanna *et al.*, 2003).

A substantial amount of eDNA can be found within the ECM of many bacterial species. It is assumed to play structural and protective roles for the biofilm community. eDNA might derive from lysed host cells as well as from lysed bacterial cells or it might be actively released from bacteria via secretion of outer membrane vesicles. Treatment of biofilms with DNAse I results in decreased viability of bacteria and reduced tolerance to environmental factors (Whitchurch *et al.*, 2002, Tetz & Tetz, 2010).

The polysaccharide adhesin PGA stabilizes *E. coli* biofilms by promoting abiotic surface binding and intercellular cohesion (Wang *et al.*, 2004). The *pgaABCD* transcript is destabilized by the RNA-binding protein CsrA (Wang *et al.*, 2005) (see chapter 5.3). Translation of *pgaA* mRNA is activated by McaS (Thomason *et al.*, 2012), a small regulatory RNA that also targets *csgD* and *flhDC* mRNAs (see chapter 5.3). PGA production is allosterically activated by c-di-GMP which binds to the PGA synthesis machinery thereby promoting enzyme activity (Steiner *et al.*, 2013). In the microarray, *pgaA* expression was found to be downregulated by YjjQ 3.1-fold in *E. coli* K-12 and 2.9-fold in UPEC strain CFT073. The possible manipulation of the three described substances by YjjQ and how this manipulation may affect morphotype expression remains unknown.

3.4 Motility assay

YjjQ was previously implicated to play a role in the negative regulation of motility in UPEC strain CFT073 since transposon mutants with restored swimming motility carried a disruption in *yjjQ* (Simms & Mobley, 2008). This putative involvement of YjjQ in the repression of motility was confirmed by the microarray data analysis in UPEC strain KEC394 (CFT073 Δ(*yjjP-yjjQ-bglJ*)::KD3). In this strain, the *flhDC* locus encoding the master regulator of flagellar motility was 9.8-fold downregulated by YjjQ. The fact that there was no differential regulation of *flhDC* expression in *E. coli* K-12 can be explained by the properties of the used strain. The microarray was performed in *E. coli* K-12 strain S3922 (BW30270 Δ(*yjjP-yjjQ-bglJ*)::KD3) which is non-

motile. The *flhDC* locus is assumed not to be expressed and YjjQ therefore has no influence on S3922 motility.

Altered swimming behavior assessed with a motility assay does not give any information about what level of the flagellar regulon is affected as a consequence of expression of the protein of interest. A reduced motility could result from impaired flagella synthesis or from paralysis of existing flagella. The microarray performed in UPEC strain CFT073 detected YjjQ-mediated repression of the flagellar regulon at the top of the hierarchy. *flhDC* is a class I operon whose expression is necessary to trigger the transcriptional cascade activating classes II and III. Therefore it is reasonable to argue that the reduced motility of transformant strains overexpressing YjjQ is due to repression of flagella synthesis rather than activity. To test this hypothesis, the activity of promoter-*lacZ* fusions was examined in the presence of YjjQ (see below, chapter 3.6). Transcription from the *flhDC* promoter was repressed by moderate YjjQ protein levels. This implies that the transcriptional repressor YjjQ directly binds to the *flhDC* promoter region thereby suppressing motility.

A possible approach to clarify if YjjQ actually represses motility by targeting *flhDC* is a mutagenesis study. Putative binding sites of YjjQ in the *flhDC* regulatory region could be mutated in an *E. coli* strain expressing YjjQ and then used in a motility assay. This strain should be motile because YjjQ-mediated repression is lost due to the disrupted sequence. If this is the case, YjjQ does in deed target the *flhDC* promoter and not one of the later expressed class II or class III flagellar promoters.

3.5 Investigation of UPEC adhesion with the help of *in vitro* model systems

In order to assess the adhesion capacities of different *E. coli* strains, two types of adhesion assays were performed. Both methods were used to examine the effects of defective curli and/or cellulose production as well as of YjjQ expression on the adherence of UPEC to eukaryotic cells. Deletion of the genes responsible for curli and/or cellulose production was expected to cause severe adhesion defects because these two main ECM components largely contribute to biofilm formation. Since YjjQ was found to substantially repress the *bcs* locus

dedicated to the synthesis of the exopolysacchriade cellulose in both *E. coli* strains tested (Table 3), overexpression of YjjQ was as well anticipated to reduce bacterial adhesion.

For the classical adhesion assay the UPEC strain CFT073 was used, a strain that was previously shown to neither produce curli nor cellulose (Hancock et al., 2007). That is why deletion of *bcsA*, *csgA* and/or *csgD* should have no effect on the adherence of CFT073 to urothelial 5637 cells. But as described in chapter 2.6.1, deletion of these genes as well as overexpression of YjjQ increased the overall colony numbers of CFT073. The reason for this phenomenon remains elusive. Possibly other adhesion factors such as different types of fimbriae mediate increased adherence in the deletion mutant strains, but this hypothesis is highly speculative.

The microscopy-based adhesion assay was supposed to replace the adhesion assay because it offers several advantages. For this method the UPEC strain 536 was used, a strain that exhibits the red, dry and rough (rdar) morphotype indicating co-expression of curli and cellulose. Therefore deletion of the genes responsible for curli and/or cellulose production was supposed to reduce the adhesion capacity of this strain. Furthermore, since overexpression of YjjQ led to a pronounced attenuation of the rdar morphotype in 536, YjjQ expression was likewise expected to alter adherence of this strain. Despite the successful establishment of the assay protocol, this method failed to provide an improved system for the analysis of UPEC adhesion. It seems that the types of adhesion assays that include a centrifugation step in the procedure only detect bacteria that were forced onto the surface by centrifugation. For that reason also the second cell culture-based model system used to investigate adhesion of UPEC to eukaryotic cells was abandoned.

3.6 Putative functions of YjjQ in *E. coli*

In the course of this study the transcription factor YjjQ was identified as a repressor of both motility and adhesion at the same time. YjjQ emerged as a negative regulator of flagella (*flhDC*) and a putative negative regulator of type 1 fimbriae (*fimB*), cellulose synthesis (*bcs*), capsule synthesis (*gfc*, *kpsMT*), acid stress (*adiY adiC, dctR*), and energy metabolism (*cra*, *dcuB*, *frdABCD*) (Table 4). It affects at least one of the three bacterial surface proteins strongly associated with biofilm formation: flagella, type 1 fimbriae, and curli (Prüß *et al.*, 2006). The

flhDC operon seems to be an actual YjjQ target as demonstrated in the motility assay. Regulation of some of the putative YjjQ target genes identified in the microarray such as *ucpA*, *fruBKA*, *zinT*, and *dctR* may result from indirect regulation via the repression of their transcriptional activators and repressors (see above, chapter 3.1). Therefore, it would be interesting to analyze YjjQ-mediated repression of the transcriptional regulators *adiY*, *cra*, *dctR*, *flhDC*, and *zur* (see chapter 3.1) in more detail. However, for the majority of the putative target genes identified in the microarray, the relation to YjjQ remains to be established.

Analysis of the regulation of the loci presumably repressed by YjjQ revealed that this protein has several targets in common with global transcription factors and TCSs. Some of these targets are repressed by H-NS and are regulated by the osmoregulatory response regulator OmpR as well as by the FixJ/NarL-type response regulator RcsB. OmpR is a negative regulator of flagella (Shin & Park, 1995) as well as type 1 fimbriae (Schwan *et al.*, 2002) and a positive regulator of curli (Vidal et al., 1998). RcsB is a negative regulator of flagella (Francez-Charlot et al., 2003) as well as curli (Vianney et al., 2005) and a positive regulator of type 1 fimbriae (Schwan et al., 2007). In this way the EnvZ-OmpR TCS and the RcsCDB TCS are involved in controlling the switch between motility and sessility. YjjQ could represent another important protein responsible for fine-tuning the transition between the planktonic and the biofilm lifestyle. In doing so, YjjQ seems to counteract the functions of some other FixJ/NarL-type transcription factors (Figure 28). Therefore YjjQ may represent an antagonist limiting some of the activities of RcsB homodimers and heterodimers and possibly of other family members such as CsgD.

In our laboratory efforts were already made in order to revise the microarray data with more specific methods. Chromosomal *lacZ* reporters of selected putative YjjQ target promoters were constructed in an *E. coli* K-12 strain deleted for *yjjP-yjjQ-bglJ*. These strains were transformed with the plasmids pKERV17 ($YjjQ_{K\text{-}12\varepsilon SD}$) and pKEKD31 ($YjjQ_{K\text{-}12nSD}$) (Table 13), respectively, and the influence of high and moderate YjjQ expression levels on the transcriptional activity was analyzed with β-galactosidase assays (Groß, 2013). Surprisingly, transcription from the *adiY* promoter, the gene showing the highest regulation by YjjQ in the microarray in both *E. coli* strains, is not decreased following plasmidic YjjQ expression under the experimental conditions applied. The same is true for several other putative YjjQ target loci including the cellulose

biosynthesis operons, *bcsABZC* and *bcsEFG*. However, the promoter region of *flhDC* is an actual YjjQ target as reflected by reduced transcriptional activity.

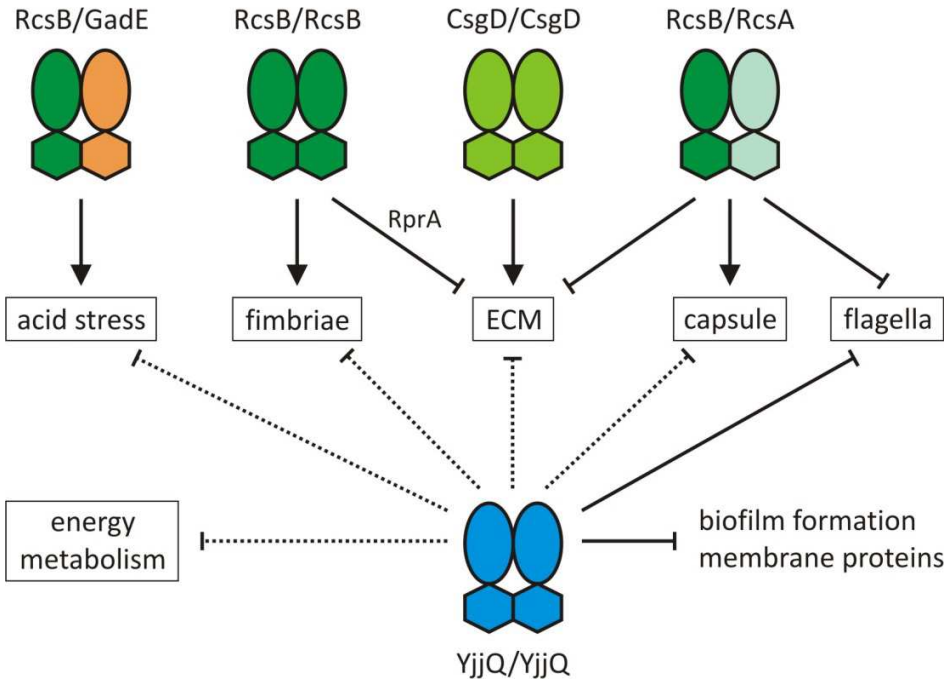


Figure 28: Putative functions of the FixJ/NarL-type transcription factor YjjQ in *E. coli*.
YjjQ putatively suppresses energy metabolism via repression of the catabolite repressor/activator protein Cra, the sRNA and CsrA antagonist CsrB, the C_4-dicarboxylate transporter DcuB, and the Fumarate reductase enzyme complex FRD. YjjQ putatively suppresses acid stress responses via repression of the Arginine-dependent acid resistance system (AdiY AdiC) and of the FixJ/NarL-type transcription factor DctR. In parallel, YjjQ may be involved in the repression of several biofilm determinants. YjjQ putatively suppresses adhesion via repression of the FimB recombinase mediating type 1 fimbriation. YjjQ putatively suppresses ECM production via repression of the cellulose biosynthesis locus. YjjQ putatively suppresses biofilm formation via repression of capsule formation (Gfc in *E. coli* K-12, KpsMT in UPEC CFT073). In fact, YjjQ suppresses motility via repression of the master regulator of flagellar motility FlhDC.

A motif search using the MEME suite program was performed in regions encompassing the transcription start sites of the genes suppressed in the β-galactosidase assays (Groß, 2013). This approach yielded a putative DNA-binding motif for YjjQ in the *flhDC* and *ompC* promoter regions. YjjQ binding in the promoter regions of these target loci could interfere with the binding of other transcriptional regulators. In the case of the *flhDC* promoter, the putative YjjQ binding site is located at the transcription start site. Thus, YjjQ binding could prevent access of

the RNA polymerase to the promoter. In the case of the *ompC* promoter, the putative YjjQ binding site lies upstream of the transcription start site. It overlaps with one of three OmpR binding sites, an osmoregulatory response regulator known to be involved in the regulation of the *ompC* locus (Yoshida *et al.*, 2006) (see chapter 2.2).

The computed DNA-binding motif is partly palindromic which strongly supports the notion of YjjQ being active as a homodimer (Groß, 2013). Further experiments to elucidate the functions of YjjQ could include mutagenesis of this putative DNA-binding motif.

4. Material and Methods

4.1 Material

4.1.1 Bacterial strains, plasmids, and oligonucleotides

Escherichia coli K-12 strains used in this study are listed in Table 10, uropathogenic *Escherichia coli* strains are listed in Table 11 and Table 12, respectively. Plasmids are summarized in Table 13, and sequences of oligonucleotides are given in Table 14. Strains were stored as dimethylsulfoxide (DMSO) stocks at -80°C.

Table 10: *Escherichia coli* K-12 strains

Strain	Relevant genotype	Reference/Construction
BTH101	F-, cya-99, araD139, galE15, galK16, rpsL1 (Str r), hsdR2, mcrA1, mcrB1 (stored as S4135)	(Karimova et al., 1998)
BW23474	F-, Δ(argF-lac)169, ΔuidA4::pir-116, recA1, rpoS396(Am)?, endA9(del-ins)::FRT, rph-1, hsdR514, rob-1, creC510 CGSC # 7838 (stored as T585)	(Haldimann *et al.*, 1998)
BW30270	MG1655 rph^{+}, CGSC # 7925 (motile) (stored as S3839)	Coli Genetic Stock Center #7925
DH5α	supE44 ΔlacU159 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (stored as S103)	(Xia *et al.*, 2011)
FW102	CSH142 strepR / F´ Kan OL2-62 lac (Stored as S3773)	(Whipple, 1998)
MG1655	K-12 wild-type (stored as S3836)	(Guyer *et al.*, 1981)
S3440	attB::(SpecR $lacI^{q}$ rrnB-T1 PsulA lexA-Op408/wt lacZ) sulA3 lexA71::Tn5 ΔrcsB::FRT	(Venkatesh et al., 2010)
S3442	attB::(SpecR $lacI^{q}$ rrnB-T1 PsulA lexA-Op408/wt lacZ) sulA3 lexA71::Tn5 Δ(yjjP-yjjQ-bglJ)::FRT ΔrcsB::FRT	(Venkatesh et al., 2010)
S3922	BW30270 Δ(yjjP-yjjQ-bglJ)::KD3-cm, non-motile	laboratory collection
T1241	BW30270 ilvG+ (motile)	laboratory collection
T66	BW30270 Plac::gfp-mut1 ΔlacZYA-kan, non-motile	laboratory collection
XL1-Blue	F'::Tn10 (TetR) proA+B+ $lacI^{q}$ Δ(lacZ)M15 / recA1 endA1 gyrA96 (NalR) thi-1 hsdR17 (rk–mk–) glnV44 relA1 lac (stored as S3984)	(Xia et al., 2011)

Table 11: Uropathogenic *Escherichia coli* CFT073 strains

Strain	**Relevant genotype**	**Reference/Construction**[a]
CFT073	UPEC obtained from Dr. U. Dobrindt, Würzburg (stored as KEC375)	(Welch et al., 2002)
KEC394	CFT073 Δ(yjjP-yjjQ-bglJ)::KD3	laboratory collection
KEC466	CFT073 Δ*bcsA*::KD3	KEC465 × PCR T445/T446 (pKD3)
KEC467	CFT073 Δ*bcsA*::KD4	KEC465 × PCR T445/T446 (pKD4)
KEC468	CFT073 Δ*csgA*::KD3	KEC465 × PCR T449/T450 (pKD3)
KEC469	CFT073 Δ*csgA*::KD4	KEC465 × PCR T449/T450 (pKD4)
KEC470	CFT073 Δ*csgD*::KD3	KEC465 × PCR T453/T454 (pKD3)
KEC471	CFT073 Δ*csgD*::KD4	KEC465 × PCR T453/T454 (pKD4)
KEC472	CFT073 Δ*bcsA*::KD3 Δ*csgA*::KD4	KEC466+pKD46 × PCR T449/T450 (pKD4)

a) Construction of UPEC CFT073 strains by λ-Red mediated recombination (x PCR) briefly described below was performed as described previously (Datsenko & Wanner, 2000).

Table 12: Uropathogenic *Escherichia coli* 536 strains

Strain	Relevant genotype[b]	Reference/Construction[a]
536	UPEC obtained from Dr. U. Dobrindt, Würzburg (stored as KEC293)	(Brzuszkiewicz et al., 2006)
KEC478	536 Δ*bcsA*::KD3	KEC475 × PCR T445/T446 (pKD3)
KEC479	536 Δ*csgA*::KD3	KEC475 × PCR T449/T450 (pKD3)
KEC481	536 Δ*csgD*::KD3	KEC475 × PCR T453/T454 (pKD3)
KEC482	536 Δ(*yjjP-yjjQ-bglJ*)::KD3	KEC475 × PCR S783/S676 (pKD3)
KEC483	536 Δ(*yjjQ-bglJ*)::KD3	KEC475 × PCR T578/S676 (pKD3)
KEC484	536 Δ*bcsA*::FRT	KEC478 × pCP20
KEC485	536 Δ*csgA*::FRT	KEC479 × pCP20
KEC486	536 Δ(*yjjQ-bglJ*)::FRT	KEC483 × pCP20
KEC487	536 Δ(*yjjP-yjjQ-bglJ*)::FRT	KEC482 × pCP20
KEC488	536 Δ*csgD*::FRT	KEC481 × pCP20
KEC489	536 Δ*bglJ*::KD3	KEC475 × PCR S675/S676 (pKD3)
KEC490	536 Δ*lacA*::$P_L$gfp-KD3	KEC475 × PCR T683/T684 (pKD3)
KEC491	536 Δ*bcsA*::FRT Δ*csgA*::KD3	KEC484 × EB49[KEC479]
KEC492	536 Δ*csgA*::FRT Δ*bcsA*::KD3	KEC485 × EB49[KEC478]
KEC493	536 Δ*csgA*::FRT Δ*bcsA*::KD3	KEC485 × EB49[KEC478]
KEC494	536 Δ*bglJ*::FRT	KEC489 × pCP20
KEC495	536 Δ*csgA*::FRT Δ*bcsA*::FRT	KEC492 × pCP20
KEC496	536 Δ*bcsA*::FRT Δ*csgA*::FRT	KEC491 × pCP20
KEC497	536 Δ*bcsA*::FRT Δ*lacA*::$P_L$gfp-KD3	KEC484 × EB49[KEC490]
KEC498	536 Δ*csgA*::FRT Δ*lacA*::$P_L$gfp-KD3	KEC485 × EB49[KEC490]
KEC499	536 Δ(*yjjQ-bglJ*)::FRT Δ*lacA*::$P_L$gfp-KD3	KEC486 × EB49[KEC490]
KEC500	536 Δ(*yjjP-yjjQ-bglJ*)::FRT Δ*lacA*::$P_L$gfp-KD3	KEC487 × EB49[KEC490]
KEC501	536 Δ*csgD*::FRT Δ*lacA*::$P_L$gfp-KD3	KEC488 × EB49[KEC490]
KEC502	536 Δ*bglJ*::FRT Δ*lacA*::$P_L$gfp-KD3	KEC494 × EB49[KEC490]
KEC503	536 Δ*bcsA*::FRT Δ*csgA*::FRT Δ*lacA*::$P_L$gfp-KD3	KEC496 × EB49[KEC490]
KEC504	536 Δ*csgA*::FRT Δ*bcsA*::FRT Δ*lacA*::$P_L$gfp-KD3	KEC495 × EB49[KEC490]

a) Construction of UPEC 536 strains by λ-Red mediated recombination (x PCR), by Flp mediated recombination (x pCP20), or by phage transduction (x EB49) briefly described below were performed as described previously (Datsenko & Wanner, 2000, Cherepanov & Wackernagel, 1995, Battaglioli et al., 2011).

b) UPEC 536 strain KEC490 and its descendants KEC497 to KEC504 harbor *gfp-mut1* (Cormack et al., 1996) derived from pKEIB16 (Table 13).

Table 13: Plasmids

Plasmid	Features/Use[c]	Reference
pDOC-G	sacB bla neo template plasmid with GFP tag for Gene Doctoring	(Lee *et al.*, 2009)
pFDX334	p15A-ori, neo, cI-857 DH5α transformant stored as strain T1155	Rak laboratory, Freiburg
pCP20	cI_{857} λ-P_R flp in pSC101 rep^{ts} bla cat	(Cherepanov & Wackernagel, 1995)
pKD3	FRT-cat - FRT oriRγ bla	(Datsenko & Wanner, 2000)
pKD4	FRT-neo - FRT oriRγ bla	(Datsenko & Wanner, 2000)
pKD46	araC P_{ara} γ-β-exo in pSC101 rep^{ts} bla CFT073 transformant stored as strain KEC465 536 transformant stored as strain KEC475 KEC478 transformant stored as strain KEC480	(Datsenko & Wanner, 2000)
pKES286	pKD3 with MCS	laboratory collection
pDP804	P_{lacUV5} (lacO1) $lexA_{408}$-Jun bla, positive control for LexA two-hybrid	(Venkatesh et al., 2010)
pMS604	P_{lacUV5} (lacO1) $lexA_{wt}$-Fos tet, positive control for LexA two-hybrid	(Venkatesh et al., 2010)
pAC λcI	P_{lacUV5}, p15A-ori, cat, negative control for cI-α two-hybrid	(Dove & Hochschild, 2004)
pAC λcI-β 831-1057	P_{lacUV5}, p15A-ori, cat, cloning vector for cI-α two-hybrid	(Dove & Hochschild, 2004)
pBR α	P_{lacUV5}, pBR-ori, bla, negative control for cI-α two-hybrid	(Dove & Hochschild, 2004)
pBR α-β 831-1057	P_{lacUV5}, pBR-ori, bla, cloning vector for cI-α two-hybrid	(Dove & Hochschild, 2004)
pBR α-σ^{70} D581G	P_{lacUV5}, pBR-ori, bla, positive control for cI-α two-hybrid	(Dove & Hochschild, 2004)
p25-N	N-terminal fusion to the T25 fragment for BACTH, P_{lac} neo	(Karimova et al., 1998)
pKT25	C-terminal fusion to the T25 fragment for BACTH, P_{lac} neo	(Karimova et al., 1998)
pKT25-zip	positive control for BACTH the T25 fragment of CyaA fused to a Leu-zipper, P_{lac} neo	(Karimova et al., 1998)
pUT18	N-terminal fusion to the T18 fragment for BACTH, P_{lac} bla	(Karimova et al., 1998)
pUT18C	C-terminal fusion to the T18 fragment for BACTH, P_{lac} bla	(Karimova et al., 1998)
pUT18C-zip	positive control for BACTH the T18 fragment of CyaA fused to a Leu-zipper, P_{lac} bla	(Karimova et al., 1998)
pKEAP27	lexA-$WT_{(1-87)}$-yjjQ, P_{lacUV5} tet	(Venkatesh et al., 2010)
pKEAP28	lexA-$408_{(1-87)}$-rcsB, P_{lacUV5} bla	(Venkatesh et al., 2010)
pKEAP29	lexA-$408_{(1-87)}$-bglJ, P_{lacUV5} bla	(Venkatesh et al., 2010)
pKEAP30	lexA-$WT_{(1-87)}$-bglJ, P_{lacUV5} tet	(Venkatesh et al., 2010)
pKEIB16	$lacI^q$ tacOP gfp-mut1 bla	laboratory collection
pKEMK1	lexA-$WT_{(1-87)}$-dctR, P_{lacUV5} tet	laboratory collection
pKEMK4	lexA-$WT_{(1-87)}$-matA, P_{lacUV5} tet	laboratory collection
pKEMK15	lexA-$WT_{(1-87)}$-evgA, P_{lacUV5} tet	laboratory collection
pKEMK16	lexA-$WT_{(1-87)}$-gadE, P_{lacUV5} tet	laboratory collection
pKEMK17	lexA-$WT_{(1-87)}$-rcsB, P_{lacUV5} tet	(Venkatesh et al., 2010)

Plasmid	Features/Use[c]	Reference
pKERV17	*lacI*^{q} P_{tac} (εSD) *yjjQ* (K-12) p15A-ori neo	laboratory collection
pKES169	*lacI*^{q}, P_{tac}, p15A-ori, neo vector control for pKEKD25 transformant used in the adhesion assay	laboratory collection
pKES192	lexA-$WT_{(1-87)}$-rcsA, P_{lacUV5} tet	(Venkatesh et al., 2010)
pKESK22	*lacI*^{q}, P_{tac}, p15A-ori, neo vector control for pKERV17, pKEKD25, 30, 31 transformants	(Stratmann et al., 2008)
pKEKD1	pUT18C-bglJ for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T432	this work
pKEKD2	pUT18C-dctR for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T428	this work
pKEKD3	pUT18C-evgA for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T429	this work
pKEKD4	pUT18C-gadE for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T430	this work
pKEKD5	pUT18C-matA for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T433	this work
pKEKD6	pUT18C-rcsA for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T434	this work
pKEKD7	pUT18C-rcsB for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T431	this work
pKEKD8	pUT18C-yjjQ for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T435	this work
pKEKD9	pKT25-bglJ for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T460	this work
pKEKD10	pKT25-dctR for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T464	this work
pKEKD11	pKT25-evgA for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T465	this work
pKEKD12	pKT25-gadE for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T466	this work
pKEKD13	pKT25-matA for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T467	this work
pKEKD14	pKT25-rcsA for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T461	this work
pKEKD15	pKT25-rcsB for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T462	this work
pKEKD16	pKT25-yjjQ for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T463	this work
pKEKD17	pUT18-rcsA for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T549	this work
pKEKD18	pUT18-rcsB for BACTH, P_{lac} bla XL1-Blue transformant stored as strain T550	this work
pKEKD19	p25-N-rcsA for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T551	this work

Plasmid	Features/Use[c]	Reference
pKEKD20	p25-N-rcsB for BACTH, P_{lac} neo XL1-Blue transformant stored as strain T552	this work
pKEKD21	pKES189-evgA for LexA two-hybrid, P_{lacUV5} bla XL1-Blue transformant stored as strain T726	this work
pKEKD22	pKES189-gadE for LexA two-hybrid, P_{lacUV5} bla XL1-Blue transformant stored as strain T670	this work
pKEKD23	pAC-λ-cI-β-rcsB for cI-α two-hybrid, P_{lacUV5} cat XL1-Blue transformant stored as strain T756	this work
pKEKD24	pBR-α-β-rcsB for cI-α two-hybrid, P_{lacUV5} bla XL1-Blue transformant stored as strain T797	this work
pKEKD25	*lacI*^{q} P_{tac} (εSD) *yjjQ* (CFT073) p15A-ori neo XL1-Blue transformant stored as strain T933 CFT073 transformant stored as strain KEC473 536 transformant stored as strain KEC476	this work
pKEKD26	gfp-mut1 (εSD) from pKEIB16 in pDOC-G XL1-Blue transformant stored as strain T1007	this work
pKEKD27	P_L gfp-mut1 (εSD) in pKEKD26 XL1-Blue pFDX334 co-transformant stored as strain T1026	this work
pKEKD28	P_L gfp-mut1 (εSD) lacA HR2 in pKEKD27 XL1-Blue transformant stored as strain T1057	this work
pKEKD29	P_L gfp-mut1 (εSD) lacA HR2+HR1 in pKEKD28 XL1-Blue pFDX334 co-transformant stored as strain T1065 XL1-Blue transformant stored as strain T1070	this work
pKEKD30	*lacI*^{q} P_{tac} *yjjQ* (536) p15A-ori neo XL1-Blue transformant stored as strain T1105	this work
pKEKD31	*lacI*^{q} P_{tac} *yjjQ* (K-12) p15A-ori neo XL1-Blue transformant stored as strain T1113	this work
pKEKD32	P_L gfp-mut1 (εSD) in pKES286 BW23474 pFDX334 co-transformant stored as strain T1153	this work

c) Plasmid pFDX334 encodes the temperature-sensitive λ-cI-857 repressor that represses the P_L promoter of phage λ at low temperatures (George et al., 1987). Plasmids pKERV17 and pKEKD25 harbor *yjjQ* from *E. coli* strain K-12 and UPEC strain CFT073, respectively, preceded by the enhanced epsilon Shine-Dalgarno sequence (εSD) derived from the leader of gene 10 in phage T7 (Olins et al., 1988). Plasmids pKEKD30 and pKEKD31 harbor *yjjQ* from UPEC strain 536 and *E. coli* strain K-12, respectively, preceded by the native Shine-Dalgarno sequence. Plasmids pKEKD26 to pKEKD29 and pKEKD32 encode *gfp-mut1* (Cormack et al., 1996) derived from pKEIB16 and preceded by εSD.

Table 14: Oligonucleotides

Oligo	Sequence[d]	Target/application
S116	TGGCACGACAGGTTTCCCGA	sequencing of pKEKD25, 30, 31
S117	CTTTATGCTTCCGGCTCGTA	sequencing of pKEKD21, 22, 27, 28
S123	TGTGGAATTGTGAGCGGATA	sequencing of pKEKD26
S258	TCATTTATTCTGCCTCCCAGAGC	cloning of pKEKD24; sequencing of pKEKD23
S323	ggcccATATGTTGCCAGGATGCTGCAA	cloning of pKEKD25, NdeI site
S358	CGGTATCAACAGGGACACCAGGATTTATTTATTCT	sequencing of pKEKD32
S675	ACAGCCGAATTAAGAAGAGAAATGTCGCACTCATAGAAAAATGCGTC**gtg taggctggagctgcttcg**	*bglJ* deletion in UPEC 536
S676	GAAATGAAAGCACTGCCGGGGAAGTAAACCCGGCATCATGCGGATTA**ca tatgaatatcctccttagttcctattcc**	*bglJ*/(*yjjQ-bglJ*)/(*yjjP-yjjQ-bglJ*) deletions in UPEC 536
S707	GAATGACATCACCTTCCTCCACC	sequencing of pKEKD21, 22
S783	GAGGATCATATCCTGCGCCAACGCTAACAGAAATTCGATCA**gtgtaggctgg agctgcttcg**	(*yjjP-yjjQ-bglJ*) deletion in UPEC 536
S786	CCAGGTAATGATTTACAGCGGCAAG	analysis of *lacA* replacement in UPEC KEC490, 497-504
S795	AACACCATCGCAAAGCCGAC	analysis of (*yjjP-yjjQ-bglJ*) deletion in UPEC KEC482
S873	TTCTGACACATGCAGTGGAGTTGTT	analysis of (*yjjQ-bglJ*) deletion in UPEC KEC483
S920	TGCCAGCGTTATCGCTTACC	analysis of *bglJ*/(*yjjQ-bglJ*)/(*yjjP-yjjQ-bglJ*) deletions in UPEC KEC482, 483, 489
T24	GAAAAGTGCCACCTGACGTCTAA	sequencing of pKEKD1-8
T95	cagtctagattaTGAGTGCGACATTTCTCTTCTTAATTC	cloning of pKEKD25, 30, 31, XbaI site
T106	cagggatcctctagaTTAGTCTTTATCTGCCGGACTTAAGGTCAC	cloning of pKEKD23, BamHI + XbaI site
T123	AGCGCAACGCAATTAATGTGAGTTAGCTCA	sequencing of pKEKD17-20, 26
T124	TCGCTATTACGCCAGCTGGCGAAAG	sequencing of pKEKD9
T204	TGGCGCTGGGCTTCACCTT	analysis of *lacA* replacement in UPEC KEC490, 497-504; sequencing of KEC490
T292	GCGAGGGCTATGTCTTCTACGA	sequencing of pKEKD1-8
T293	gatatctagaaGAACACAGCCGAATTAAGAAGAGAA	cloning of pKEKD1, XbaI site
T294	gataggtaccTTAATAGGGATGCAACACATTACTTGTT	cloning of pKEKD1, KpnI site
T295	gatatctagaaTTTCTTATAATTACCAGGGATACGATGTT	cloning of pKEKD2, XbaI site
T296	gataggtaccTCACACCAGATAATCAATATGCTGATG	cloning of pKEKD2, KpnI site
T297	gatatctagaaAACGCAATAATTATTGATGACCATCC	cloning of pKEKD3, XbaI site
T298	gataggtaccTTAGCCGATTTTGTTACGTTGTGC	cloning of pKEKD3, KpnI site
T299	gatatctagaaATTTTTCTCATGACGAAAGATTCTTTTC	cloning of pKEKD4, XbaI site
T300	gataggtaccCTAAAAATAAGATGTGATACCCAGGGTG	cloning of pKEKD4, KpnI site
T301	gatatctagaaACATGGCAAAGTGATTACAGTAGGG	cloning of pKEKD5, XbaI site

Oligo	Sequence[d]	Target/application
T302	gataggtaccTTACTGAACCAACTTATATATTTTTGAGTACAGC	cloning of pKEKD5, KpnI site
T303	gatatctagaaTCAACGATTATTATGGATTTATGTAGTTACAC	cloning of pKEKD6, 17, 19, XbaI site
T304	gataggtaccTTAGCGCATGTTGACAAAAATACC	cloning of pKEKD6, KpnI site
T305	gatatctagaaAACAATATGAACGTAATTATTGCCGA	cloning of pKEKD7, 18, 20, XbaI site
T306	gataggtaccTTAGTCTTTATCTGCCGGACTTAAGG	cloning of pKEKD7, KpnI site
T307	gatatctagaaTTGCCAGGATGCTGCAAAA	cloning of pKEKD8, XbaI site
T308	gataggtaccCTATGAGTGCGACATTTCTCTTCTTAAT	cloning of pKEKD8, KpnI site
T363	GTCGTAGCGGAACTGGCG	sequencing of pKEKD17, 18
T364	ATCAATGTGGCGTTTTTTTCCTT	sequencing of pKEKD19, 20
T365	gataggtaccgcGCGCATGTTGACAAAAATACCA	cloning of pKEKD17, 19, Acc65I site
T366	gataggtaccgcGTCTTTATCTGCCGGACTTAAGGTC	cloning of pKEKD18, 20, Acc65I site
T407	gatactcgagAACGCAATAATTATTGATGACCATCC	cloning of pKEKD21, XhoI site
T408	gataagatctTTAGCCGATTTTGTTACGTTGTGC	cloning of pKEKD21, BglII site
T409	gatactcgagATTTTTCTCATGACGAAAGATTCTTTTC	cloning of pKEKD22, XhoI site
T410	gataagatctCTAAAAATAAGATGTGATACCCAGGGTG	cloning of pKEKD22, BglII site
T420	ATGGAACACAGCCGAATTAAGAAGAG	analysis of *bglJ* deletion in UPEC KEC489
T426	gatagcggccgcaAACAATATGAACGTAATTATTGCCGA	cloning of pKEKD23, NotI site
T427	GGGATAGCGGTCAGGTGTTTTT	cloning of pKEKD24; sequencing of pKEKD23
T428	GGAAACCAACGGCACAATCG	sequencing of pKEKD24
T429	TACCCACGCCGAAACAAGC	sequencing of pKEKD24
T430	TGTCCTACTCAGGAGAGCGTTCAC	sequencing of pKEKD29
T445	CGGTGGTTGCTTATCCCGCCGGTCAACGCACGGCTTATC**gtgtaggctggagc tgcttcg**	*bcsA* deletion in UPEC CFT073 and 536
T446	CTTTTCATCGCGTTATCATCATTGTTGAGCCAAAGCCTG**catatgaatatcctc cttagttcctattcc**	*bcsA* deletion in UPEC CFT073 and 536
T447	GATTTTGCTCCGCCTGGG	analysis of *bcsA* deletion in UPEC KEC466, 467, 478, 491-493
T448	CGATGGCTGAATGTCTGGCA	analysis of *bcsA* deletion in UPEC KEC466, 467, 478, 491-493
T449	ATCCGATGGGGGTTTTACATGAAACTTTTAAAAGTAGCAGCA**gtgtaggctg gagctgcttcg**	*csgA* deletion in UPEC CFT073 and 536
T450	TTTCATACTGATGATGTATTAGTACTGATGAGCGGTCGCGTT**catatgaata tcctccttagttcctattcc**	*csgA* deletion in UPEC CFT073 and 536
T451	TGCCAACGATGCCAGTATTTC	analysis of *csgA* deletion in UPEC KEC468, 469, 472, 479, 491-493
T452	TGGTGTACATATCCCCTTGCTGG	analysis of *csgA* deletion in UPEC KEC468, 469, 472, 479, 491-493
T453	AAAAAGCGGGGTTTCATCATGTTTAATGAAGTCCATAGTATT**gtgtaggctg gagctgcttcg**	*csgD* deletion in UPEC CFT073 and 536
T454	TTATCGCCTGAGGTTATCGTTTGCCCAGGAAACCGCTTGTGT**catatgaata tcctccttagttcctattcc**	*csgD* deletion in UPEC CFT073 and 536

Oligo	Sequence[d]	Target/application
T455	ATCACTAAAGGCTCGGTAAAAATCAT	analysis of *csgD* deletion in UPEC KEC470, 471, 481
T456	ATCCATTAGTTTTATATTTTACCCATTTAGG	analysis of *csgD* deletion in UPEC KEC470, 471, 481
T534	gataggtaccAATAATTTTGTTTAACTTTAAGAAGGAGATATACA	cloning of pKEKD26, Acc65I site
T535	gataaccggtTTATTTGTATAGTTCATCCATGCCATG	cloning of pKEKD26, AgeI site
T536	gataggtaccTCATGGTGGTCAGTGCGTCC	cloning of pKEKD27, Acc65I site
T537	gataggatccCTCTCACCTACCAAACAATGCCC	cloning of pKEKD27, 32, BamHI site
T542	TTCTACGTGTTCCGCTTCCTTTA	sequencing of pKEKD27
T559	AATTCCGCCTTATCCGACCAACATATCAGGGCGGAATGATCGCATAAG	cloning of pKEKD28, lacA homology region, 5'-phosphate, EcoRI + BamHI site
T560	GATCCTTATGCGATCATTCCGCCCTGATATGTTGGTCGGATAAGGCGG	cloning of pKEKD28, lacA homology region, 5'-phosphate, EcoRI + BamHI site
T561	TCGAGTATTTCAAAGATTTTAAAGTTGAGTCTTCAGTTTAAATAAG	cloning of pKEKD29, lacA homology region, 5'-phosphate, XhoI + SalI site
T562	TCGACTTATTTAAACTGAAGACTCAACTTTAAAATCTTTGAAATAC	cloning of pKEKD29, lacA homology region, 5'-phosphate, XhoI + SalI site
T578	TAAGGATAATTTATTCGCTTAATCTATTAATTTGCTGGGAATATTTAAGG**g tgtaggctggagctgcttcg**	(*yjjQ-bglJ*) deletion in UPEC 536
T608	gatagaattcAGACAGTGGATGTGGAGGAAATATG	cloning of pKEKD30, 031, BamHI site
T621	gataggatccaccggtTTATTTGTATAGTTCATCCAT	cloning of pKEKD32, BamHI + AgeI site
T683	TTATTTAAACTGAAGACTCAACTTTAAAATCTTTGAAATA**gtgtaggctggag ctgcttcg**	*lacA* replacement in UPEC 536
T684	CGCCTTATCCGACCAACATATCAGGGCGGAATGATCGCAGGATCCCTCTC ACCTACCAAACA	*lacA* replacement in UPEC 536 (modified Datsenko-Wanner), BamHI site

d) Oligonucleotides are given in 5' to 3' direction. Matching parts to the indicated targets are printed in upper case, non-matching parts in lower case letters. Sites for restriction endonucleases are underlined and Datsenko-Wanner primer parts are printed in bold. Oligonucleotides were synthesized by Life Technologies (Invitrogen), Karlsruhe, Germany, or Sigma-Aldrich, Taufkirchen, Germany.

4.1.2 Media, buffers and antibiotics

LB medium
10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl, ad 1 l H_2O

LB agar plates
LB medium with 1.5% (w/v) Bacto agar

LB X-gal agar plates
LB medium with 1.5% (w/v) Bacto agar, 40 µg/ml X-gal

5-Brom-4-chlor-3-indoxyl-β-D-galactopyranoside (X-gal)
Stock: 20 mg/ml in Dimethylformamide
Use: 40 µg/ml for plates

Maltose/lactose MacConkey agar plates
40 g Difco MacConkey agar base, ad 1 l H_2O, after autoclaving add 1% maltose or lactose (filter sterilized)

Isopropyl-β-D-thiogalactopyranoside (IPTG)
Stock: 100 mM in H_2O (filter sterilized)
Use: 0.2 mM for plates; 1 mM for cultures

LB soft agar plates
LB medium with 0.25% (w/v) Bacto agar

L broth medium
10 g Bacto tryptone, 5 g Bacto yeast extract, 0.5 g NaCl, ad 1 l H_2O

L broth agar plates
L broth medium with 1.5% (w/v) Bacto agar

L broth top agar
L broth medium with 0.7% (w/v) Bacto agar

Phage buffer
10 mM Tris-HCl [pH 7.4], 10 mM $MgSO_4$, 0.01% (w/v) Gelatin

SOB medium
20 g Bacto tryptone, 5 g Bacto yeast extract, 0.5 g NaCl, 1.25 ml 2M KCl, adjust pH to 7.0 with NaOH, ad 1 l H_2O, after autoclaving add 10 ml 1 M $MgCl_2$

SOC medium
Add 19.8 ml 20% Glucose to 1 l SOB

TE buffer
10 ml 1 M Tris-HCl [pH8.0], 2 ml 0.5 M EDTA (ethylenediaminetetraacetic acid), ad 1 l H_2O

TEN buffer
20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 50 mM NaCl

50x TAE buffer
242 g Tris, 100 ml 0.5 M Na_2EDTA [pH 8.0], 57.1 ml glacial acetic acid, ad 1 l H_2O

Cellulose indicator plates (without salt)
10 g Bacto tryptone, 5 g Bacto yeast extract, 15 g Bacto agar, 50 µg/ml Fluorescent Brightener 28 (Calcofluor White), ad 1 l H_2O

Morphotype indicator plates (without salt)
10 g Bacto tryptone, 5 g Bacto yeast extract, 15 g Bacto agar, ad 1 l H_2O, after autoclaving add 2% (w/v) Congo Red solution

Congo Red solution
2 mg/ml Congo Red, 1 mg/ml Brilliant Blue G, dissolve in 70% (v/v) ethanol

Z buffer, pH 7.0
60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 100 µg/ml chloramphenicol

2-Nitrophenyl β-D-galactopyranoside (ONPG) buffer
4 mg/ml in 0.1 M phosphate buffer [pH 7.0] (60 mM Na_2HPO_4 + 40 mM NaH_2PO_4)

10x phosphate-buffered saline (PBS) buffer, pH 7.4
2 g KCl, 80 g NaCl, 17.8 g $Na_2HPO_4 \bullet 2H_2O$, 2.4 g KH_2PO_4, ad 1 l H_2O

Antibiotic	Stock solution	Storage temperature	Final concentration
ampicillin	50 mg/ml in 50% Ethanol	-20°C	50 µg/ml
chloramphenicol	30 mg/ml in Ethanol	-20°C	15 µg/ml
kanamycin	10 mg/ml in H_2O	4°C	25 µg/ml
streptomycin	50 mg/ml in H_2O	-20°C	50 µg/ml
tetracyclin	5 mg/ml in 70% Ethanol	-20°C	12 µg/ml

4.1.3 Eukaryotic cell culture items

5637 human urinary bladder carcinoma cell line (CLS cell lines service, Eppelheim)

RPMI 1640, with Phenol Red + GlutaMAX (contains a stabilized L-glutamine source)

Fetal Bovine Serum (FBS), EU-Approved, Standard (sterile-filtered)

0.25% Trypsin with EDTA 4Na + Phenol Red

1x Phosphate-buffered Saline (PBS) [pH 7.2]

Cell lysis solution
0.1% Triton x-100 in 1x PBS

Gentamicin protection assay (GPA) solution
Stock: 50 mg/ml gentamicin sulfate salt in H_2O
Use: 100 µg/ml in cell culture medium

Plasma membrane staining solution
Stock: 5 mg/ml CellMask Orange in DMSO
Use: 2.5 µg/ml in cell culture medium

Cell fixation solution
4% para-formaldehyde in 1x PBS (filter sterilized)

Mounting medium
Fluoroshield + DAPI

4.2 Methods

4.2.1 Standard molecular techniques

Standard molecular techniques like agarose gel electrophoresis, polymerase chain reaction (PCR), and cloning work were carried out according to published protocols (Ausubel, 2005). Supplies such as enzymes or kits were purchased from Fermentas (St. Leon-Rot, Germany), Promega (Madison, WI, USA), 5PRIME (Hamburg, Germany), and Qiagen, (Hilden, Germany). Supplies for bacterial growth media were purchased from Becton, Dickinson, and Company, Sparks, USA.
Sequencing was either performed at the Cologne Center for Genomics (CCG), University of Cologne, Germany, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) or at GATC Biotech AG, Konstanz, Germany. Sequences were analyzed using Vector NTI 11 software (Invitrogen).

Preparation of chemo-competent cells

Overnight cultures were grown in LB medium with appropriate antibiotics and at the respective temperature with shaking. From the overnight cultures 200 µl were used to inoculate 50 ml LB medium with appropriate antibiotics. The cells were grown at the respective temperature with shaking to an optical density at a wavelength of 600 nm (OD_{600}) of 0.3 and then harvested on ice. After that the culture was washed in 25 ml cold 0.1 M $CaCl_2$ followed by incubation on ice for 20 min. Finally the cells were centrifuged and the pellet was resuspended in 2 ml cold 0.1 M $CaCl_2$. 50 µl of these cells were used immediately for one transformation. Alternatively the cells were frozen for long term storage. For this purpose sterile glycerol was added to a final concentration of 15%. The cells were incubated on ice for 1 hour and aliquots of 110 µl were stored at -80°C.

Transformation

Chemo-competent cells were either used directly or thawed slowly on ice. 25 ng plasmid DNA or half of a ligation sample were prepared on ice and TEN buffer was added to a final volume of 25 µl. Then 50 µl of chemo-competent cells were added and the sample was mixed gently. The mixture was incubated on ice for 20 min. Next a heat shock was performed at 42°C for 2 min followed by incubation on ice for 10 min. After that 500 µl LB medium was added, the mixture

was transferred into a culture tube and incubated for 1 hour at 37°C with shaking. Finally various amounts were plated on selective LB agar plates and incubated overnight at the respective temperature.

Phenotypic analysis on indicator plates

BACTH assays (Karimova et al., 1998) were analyzed by two different phenotypic assays. The adenylate cyclase (*cyaA*)-deficient *E. coli* strain BTH101 was tested for revertants by streaking on LB X-gal streptomycin agar plates and incubating overnight at 37°C. An overnight culture of one Lac- or Mal- single colony was stored as DMSO stock at -80°C (50 µl of DMSO, 1.5 ml of LB overnight culture). From this stock chemo-competent cells were prepared. For blue/white-screens competent BTH101 were co-transformed with the respective plasmids and selected overnight on LB X-gal agar plates with the appropriate antibiotics containing 0.2 mM IPTG and lacking IPTG at 28°C. For red/white-screens competent BTH101 were co-transformed with the respective plasmids and selected overnight on MacConkey agar plates supplemented with 1% maltose as well as with the appropriate antibiotics at 37°C.

β-galactosidase assay

The β-galactosidase assay was used to measure promoter activities of *lacZ* reporter strains in the LexA-based and the cI-α bacterial two-hybrid assays. The method was performed as described previously (Miller, 1992). Briefly, overnight cultures were grown in LB medium with appropriate antibiotics and either supplemented with 1 mM IPTG or without IPTG at 37°C with shaking. On the next day the OD_{600} of a five-fold dilution of the overnight cultures was measured. Then exponential cultures in LB medium containing 1 mM IPTG and lacking IPTG were inoculated to an OD_{600} of 0.05 to 0.1 and grown at 37°C with shaking to an OD_{600} of 0.5. After that, the cultures were harvested on ice and three appropriate dilutions were set up in Z buffer as duplicates. To make the cells permeable one drop 0.1% (w/v) sodium dodecyl sulfate and two drops chloroform were added to all samples. Samples were pre-incubated for 10 min at 28°C, and then β-Galactosidase activities were determined by adding 200 µl of the substrate ONPG followed by incubation at 28°C. The reaction was stopped by adding 0.5 ml 1 M Na_2CO_3

and the color change was measured at OD_{420}. The assays were performed with at least three independent cultures and activity was determined according to the following formula:
1 Miller unit (MU) = [OD_{420} x dilution factor x 1000]/[OD_{600} x time (min)].

Oligonucleotide annealing

Annealing of two oligonucleotides was performed with 2 µl of complementary primers with a concentration of 10 pmol/µl. For formation of a DNA Ligase-mediated phosphodiester bond with the vector backbone the primers were ordered modified with a phosphoryl group at the 5' end. To the primer mixture 1 µl 2 M NaCl and 15 µl TE buffer were added. In the final volume of 20 µl both primers had a concentration of 1 pmol/µl. This mixture was incubated at 95°C for 5 min. After that the sample was allowed to slowly cool down to room temperature. To generate a primer concentration of 50 ng the sample was diluted appropriately and 1 µl of this dilution was used for ligation.

Preparation of electro-competent cells

Overnight cultures were grown in SOB medium with appropriate antibiotics and at the respective temperature with shaking. From the overnight cultures 200 µl were used to inoculate 50 ml SOB medium with appropriate antibiotics. The cells were grown at the respective temperature with shaking to an OD_{600} of 0.6 to 0.7 followed by incubation on ice for 1 hour. After that the culture was washed first in 50 ml cold sterile water, second in 25 ml cold sterile water, and third in 2 ml cold sterile 10% glycerol. Finally, the pellet was resuspended in 200 µl cold sterile 10% glycerol. 40 µl of these cells were used immediately for one transformation. Alternatively the cells were frozen for long term storage. For this purpose the cells were incubated on ice for 1 hour and aliquots of 40 µl were stored at -80°C.

Electroporation

Electroporation was used to introduce PCR fragments into recipient strains for chromosomal gene deletion and to introduce *E. coli* K-12-derived plasmids into UPEC strains. Electro-competent cells were either used directly or thawed slowly on ice. 0.5 µl DNA were added to one aliquot of cells, mixed and incubated for 10 min on ice. The mixture was transferred into a pre-chilled cuvette with a 0.1 cm electrode gap and pulsed at 1.8 kV for 3 sec. After that 1 ml

SOC medium was added, the mixture was transferred into a culture tube and incubated for 1 hour at 37°C with shaking. Finally, various amounts were plated on selective LB agar plates and incubated overnight at 37°C.

Gene deletion by λ-Red mediated recombination

Deletion of chromosomal genes was performed as described previously (Datsenko & Wanner, 2000). This system is based on the λ-Red mediated recombination between a linear DNA fragment and the chromosomal locus to be deleted. The basic strategy is to replace the chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR using primers with 30 to 50 base extensions homologous to the target gene.

Electro-competent cells were transformed with pKD46 and selected overnight on LB ampicillin agar plates at 28°C. Plasmid pKD46 carries a temperature-sensitive origin of replication and encodes the λ-Red system under the control of an arabinose-inducible promoter. Of these transformants electro-competent cells were generated from cultures grown at 28°C in SOB medium supplemented with ampicillin and 10 mM L-arabinose. Furthermore, a PCR using 20 ng of pKD3 or pKD4 as template was performed. Plasmids pKD3 and pKD4 encode the antibiotic resistance genes chloramphenicol and kanamycin flanked by Flp recombinase target (FRT) sites, respectively. For amplification of the PCR product primers were used that included homology regions to the chromosomal target gene as well as to the respective resistance gene. This PCR generated a fragment carrying the chloramphenicol or kanamycin resistance gene flanked by regions homologous to upstream and downstream sequences of the target gene. In addition, the FRT sites flanking the resistance gene subsequently allow its excision. The following PCR program with 30 cycles was applied for four samples with each 40 µl:

Step	Temperature	Time
1 initial denaturation	94°C	4 min
2 denaturation *	94°C	45 sec
3 annealing *	55°C	30 sec
4 elongation *	72°C	3 min
5 final elongation	72°C	10 min
6 storage	4°C	hold

* Steps 2 to 4 were repeated 29 times.

The PCR fragments were purified by agarose gel electrophoresis and eluted in water. The eluates were pooled to yield a concentration of approx. 100 ng/µl. The pKD46 transformants were transformed with 1 µl of the PCR fragment. Five times 100 µl of the transformants were plated and selected overnight at 37°C on LB chloramphenicol or kanamycin agar plates, respectively. Single colonies were re-streaked on LB chloramphenicol or kanamycin agar plates and incubated overnight at 37°C. Finally the presence of the resistance gene insertion was examined by PCR and subsequent agarose gel electrophoresis. In parallel, ampicillin sensitivity was assessed by streaking on selective agar plates to check for loss of pKD46. Overnight cultures of the deletion strains were stored as DMSO stocks at -80°C.

Resistance cassette excision by Flp recombination

Flp recombination was performed as described previously (Cherepanov & Wackernagel, 1995). Electro-competent deletion strains were transformed with pCP20 and selected overnight on LB ampicillin agar plates at 28°C. Plasmid pCP20 carries a temperature-sensitive origin of replication and encodes Flp recombinase. Flp mediated removal of the chromosomal resistance gene leaves one FRT site on the chromosome. Single colonies of putative transformants were re-streaked on LB agar plates and incubated overnight at 42°C. Ampicillin sensitivity was assessed by streaking on selective agar plates to check for loss of pCP20. Chloramphenicol or kanamycin sensitivity, respectively, was tested as well by streaking on selective agar plates. Clones sensitive for both antibiotics were examined by PCR and subsequent agarose gel electrophoresis. Overnight cultures of the deletion strains were stored as DMSO stocks at -80°C.

Generation and propagation of UPEC phage lysates

For generation of a single plaque lysate of phage EB49, 200 µl of a 536 overnight culture were mixed with 4 ml L broth top agar. The mixture was poured evenly onto fresh L broth agar plates. After that 5 µl of 10-fold serial dilutions of the initial EB49 phage stock (Battaglioli et al., 2011) ranging from 10^{0} to 10^{-10} were spotted on the overlay plates and incubated overnight at room temperature. On the next day the phages were isolated from single plaques by picking into one plaque with a pipette tip. The material was resuspended in 100 µl phage buffer and stored at 4°C.

50 µl of the isolated EB49 were mixed with 200 µl of a 536 overnight culture and 4 ml of L broth top agar. The mixture was poured evenly onto fresh L broth agar plates and incubated overnight at room temperature. On the next day the top agar was harvested and the phages were extracted with chloroform. The mixture was centrifuged to remove all remnants of soft agar. The upper phases were extracted with chloroform again by vortexing and centrifuging. The supernatants containing the phages were collected and stored at 4°C.

For titer determination, 200 µl of a 536 overnight culture were mixed with 4 ml L broth top agar. The mixture was poured evenly onto fresh L broth agar plates. After that 5 µl of 10-fold serial dilutions of the EB49 lysate ranging from 10^{-1} to 10^{-12} were spotted on the overlay plates and incubated overnight at room temperature. On the next day the plaques were counted to determine the overall titer of the stock according to the following formula:

Plaque forming units (PFUs)/ml = (number of plaques x dilution factor)/volume of the spot.

Phage transduction

Phage transduction was performed as described previously (Battaglioli et al., 2011). EB49 transduction was used to generate UPEC double mutant strains and UPEC fluorescence reporter strains. MOIs ranging from approx. 1 x 10^{-5} phage/cell to 1 x 10^{2} phage/cell were used in each transduction. 100 µl of high-titer EB49 were mixed with 500 µl of overnight cultures of the recipient 536 strain. Sodium citrate was added to a final concentration of 50 mM. After incubation at 37°C for 1 hour with shaking the mixtures were pelleted and resuspended in 500 µl L broth. 100 µl were plated and selected on L broth chloramphenicol agar plates. 100 µl of the overnight cultures, EB49 alone as well as phage buffer supplemented with sodium citrate were plated as controls. Sample and control plates were incubated overnight at 37°C. Single colonies of putative transductants were re-streaked on L broth chloramphenicol agar plates to get rid of contaminating phages and incubated overnight at 37°C. Finally the presence of the antibiotic resistance gene insertion was examined by PCR and subsequent agarose gel electrophoresis. Overnight cultures of the transduced strains were stored as DMSO stocks at -80°C.

Morphotype analysis

The Morphotype analysis was performed as described previously (Römling, 2001). The bacterial strains to be examined were streaked on respective LB agar plates and incubated overnight at 37°C. On the next day small parts of two separate single colonies of each strain were resuspended in 100 µl sterile water and vortexed briefly. 10-fold serial dilutions were made and 1 µl of the 10^{-4} and 10^{-5} dilutions of both clones was spotted in the middle of LB Congo Red agar plates. In the case of plasmidic expression, Congo Red kanamycin agar plates containing 0.2 mM IPTG and lacking IPTG were used. The plates were incubated for 8 days at 28°C and photographed afterwards.

Motility assay

E. coli K-12 strain T1241 or UPEC strain 536 was transformed with the respective plasmids and selected overnight on LB kanamycin agar plates at 37°C. LB soft agar kanamycin plates either supplemented with 0.2 mM IPTG or without IPTG were poured. In the case of pre-induction, overnight cultures were inoculated with single colonies and grown in LB medium supplemented with 1 mM IPTG or without IPTG and appropriate antibiotics at 37°C with shaking. From these cultures, 3 µl were spotted in the middle of plates containing 0.2 mM IPTG or lacking IPTG. If no pre-induction was performed, a small part of a single colony of each transformant was resuspended in 100 µl sterile water and vortexed briefly. 3 µl of these suspensions were spotted in the middle of one plate containing 0.2 mM IPTG and one lacking IPTG. The plates were incubated for five to six hours at 37°C and photographed afterwards. Swimming radii were measured and compared between transformants and control.

Biofilm assay

The biofilm assay was performed as described previously (Merritt et al., 2005). Electro-competent UPEC strain 536 and chemo-competent *E. coli* K-12 were transformed with the respective plasmids and selected overnight on LB kanamycin agar plates at 37°C. Overnight cultures were grown in LB kanamycin medium either supplemented with 1 mM IPTG or without IPTG at 37°C with shaking. The cultures were diluted 100-fold in LB kanamycin medium as well as LB kanamycin medium containing 1 mM IPTG and lacking IPTG. 100 µl of the diluted cultures

and of an LB negative control were grown in each of four wells of two 96-well microtiter plates with lid (flat bottom, polystyrene, non-tissue culture treated). The plates were incubated for 48 hours at 28°C and for 24 hours at 37°C, respectively. The bacterial suspension was removed by pipetting and the wells were washed once with 150 µl water. Then 150 µl 0.1% (w/v) crystal violet solution was added and the samples were stained for 10 min at room temperature. After that, the dye was removed by pipetting and the wells were washed twice with 200 µl water to remove any crystal violet not specifically staining the adherent bacteria. The plates were inverted and tapped vigorously on paper towels to remove any excess liquid. After the plates were air-dried, 200 µl of 30% (v/v) acetic acid was added to each stained well. The dye was solubilized by covering the plates and incubating for 15 min at room temperature.

Fluorescence microscopy

Microscopy of bacterial strains transformed with a plasmidic reporter encoding GFP-mut1 (Cormack et al., 1996) was performed with the AxioVision microscope (Zeiss). Overnight cultures of the strains to be examined were diluted to an OD_{600} of approx. 0.5 and vortexed briefly. Glass slides were coated with 500 µl of hot 1% agarose dissolved in water and covered with large cover glasses. After the agarose was cooled and solid the cover glasses were removed. 2 µl of the samples were spotted on the surface and covered with a small cover glass. Microscopy was performed using the 100-fold magnifying object lens with oil and varying exposure times.

4.2.2 Cell culture-related techniques

Supplies for eukaryotic cell culture were purchased from Life Technologies (Invitrogen), Karlsruhe, Germany, and Sigma-Aldrich, Taufkirchen, Germany.

Preparation of cell culture medium

The recommended medium for cultivation of 5637 cells is composed of RPMI 1640 medium supplemented with L-glutamine and FBS. Each new 500 ml RPMI 1640 + GlutaMAX medium bottle was supplemented with 50 ml of heat-inactivated FBS and mixed gently. The resulting RPMI 1640 + GlutaMAX containing 10% FBS is the cell culture medium that was used for all applications.

Cell counting

Determination of the cell count was performed for freezing of cells, adhesion assays, and for the preparation of microscopy samples. For cell counting a regular Neubauer counting chamber with a chamber factor of 10^4 was used. The cell count was calculated according to the following formula: Cells/ml = mean value of cells x 10^4 (x dilution factor).

Freezing and thawing of cells

For cell freezing, aliquots of 3 x 10^6 cells in 1.5 ml cell culture medium supplemented with 10% DMSO were filled in 2.0 ml cryovials. The Nalgene freezing container was used for gentle freezing at -80°C. On the next day the cryovials were transferred into a liquid nitrogen tank for long term storage. For cell thawing, each cryovial was placed in a water bath at 37°C. After thawing the cells were seeded immediately into a small flask containing pre-heated cell culture medium. On the next day cell passage was performed.

Cell passage

5637 cells were obtained from CLS Cell Lines Service GmbH (Eppelheim) at a passage number of 44. Cells were splitted every two to three days and grown to 90-100% confluence. The old cell culture medium was aspirated and the cells were washed once with PBS. After that trypsine/EDTA was added to detach the cells from the flask bottom. When the detachment process was finished cell culture medium was added to inactivate trypsin/EDTA. Cells were then

harvested, centrifuged and the supernatant was discarded. The cell pellet was resuspended in a defined volume of fresh cell culture medium. For a 2-day cultivation, cells were seeded in a 1:7 ratio. For a 3-day cultivation, cells were seeded in a 1:14 ratio. Cells were used up to a passage number of approx. 80.

Adhesion assay

The method was performed as described previously (Elsinghorst, 1994). 2.5 x 10^5 cells/ml were seeded in 6 wells per strain of a 12-well cell culture plate and grown to confluence by incubating overnight at 37°C with 5% CO_2. Furthermore, bacterial overnight cultures were inoculated in cell culture medium with appropriate antibiotics. On the next day the infection was performed. First the cell count of the eukaryotic cells was determined and the OD_{600} of the bacterial overnight cultures was measured. Exponential cultures in cell culture medium with appropriate antibiotics were inoculated to an OD_{600} of 0.1 and grown at 37°C with shaking to an OD_{600} of 0.5. In the case of overexpression studies the exponential cultures were grown to an OD_{600} of 0.3. Then 1 mM IPTG was added for induction of expression and the cultures were grown to an OD_{600} of 0.5. After that the volume of the bacterial suspension (inoculum) was calculated to adjust the desired multiplicity of infection (MOI) value (MOI = 1 means 1 bacterial cell per 1 eukaryotic cell). From the respective preparatory cultures, either the stationary overnight cultures or fresh exponential cultures induced with IPTG, the inoculums were diluted in cell culture medium and vortexed briefly. The medium was aspirated from the plate and 1 ml inoculum were added per well. The plate was centrifuged to synchronize the infection and then incubated for one to two hours at 37°C with 5% CO_2. The inoculum was aspirated and the samples were washed three times with 1 ml PBS. For cell lysis 1 ml 0.1% Triton X100 in PBS was added and the plate was incubated for 10 min at 37°C with 5% CO_2. Finally the lysates were harvested, serial dilutions were made and the two highest dilutions were plated in duplicates on LB agar plates. The inoculum suspensions were diluted and plated as well because the infection samples need to be referred to the suspensions they originated from. On the next day the colonies were counted and the evaluation of the obtained values as a graphical representation was done.

Bacterial infection of eukaryotic cells

Triplicates of 5 x 10^5 cells/ml were seeded on autoclaved round cover slips in a 6-well cell culture plate and grown overnight at 37°C with 5% CO_2. Furthermore, bacterial overnight cultures were inoculated in cell culture medium with appropriate antibiotics. On the next day the infection was performed. First the OD_{600} of the bacterial overnight cultures was measured. Exponential cultures were grown and induced as described for the adhesion assay unless otherwise indicated. After that the inoculum was calculated to adjust the desired MOI value. From the respective preparatory cultures, either the stationary overnight cultures or fresh exponential cultures induced with IPTG, the inoculums were diluted in cell culture medium and vortexed briefly. The medium was aspirated from the plate and 2 ml inoculum were added per well. The plate was centrifuged to synchronize the infection and then incubated at 37°C with 5% CO_2. After 30 min and two hours the inoculum was aspirated and the samples were washed three times with 3 ml PBS. Optional a gentamicin protection assay (GPA) was performed. Finally the samples were stained, fixed and mounted for microscopy.

Gentamicin protection assay (GPA)

The GPA was performed for determination of invasion frequencies of bacteria into eukaryotic cells. Gentamicin is a host membrane-impermeable bactericidal antibiotic that kills any extracellular bacteria. Cell culture medium was supplemented with 100 µg/ml gentamicin and vortexed briefly. The PBS was aspirated and 2 ml gentamicin medium was added per well. The cell culture plate was incubated for two hours at 37°C with 5% CO_2. After that the medium was aspirated and the samples were washed three times with 3 ml PBS. Finally the samples were stained, fixed and mounted for microscopy.

Staining, fixation and mounting procedure

A new 6-well cell culture plate was prepared with 2.5 µg/ml CellMask Orange in cell culture medium for staining of the eukaryotic plasma membrane and with 1 ml 4% para-formaldehyde in PBS for fixation. In addition, PBS for washing and sterile water for removal of salt is supplied within this plate. First the cover slip was removed from the old 6-well plate with the help of sterile tweezers. Then it was transferred into the staining solution and incubated for 1 min at

37°C. The sample was washed once with PBS, transferred into the fixation solution and incubated for 15 min at 37°C. After that the sample was washed twice with PBS. A glass slide was prepared with a drop of the mounting medium Fluoroshield which includes the nucleic acid stain DAPI. The cover slip was removed from the PBS and dipped twice into water. The residual water was absorbed with a paper towel and the cover slip was placed upside down on the mounting medium. The samples were dried for 5 min at room temperature and then stored overnight in the dark at 4°C.

Fluorescence microscopy

Microscopy of stained 5637 cells infected with bacterial reporter strains carrying a chromosomal integration of GFP-mut1 was performed with the widefield DeltaVision RT microscope (Applied Precision, Inc.) using the 40-fold magnifying object lens with oil on three fluorescence channels. The green FITC (Fluoresceinisothiocyanat) channel was used for imaging of GFP which has an excitation wavelength of 488 nm and an emission wavelength of 507 nm. The red TRITC (Tetramethylrhodaminisothiocyanat) channel was used for imaging of CellMask Orange which has an excitation wavelength of 554 nm and an emission wavelength of 567 nm. The blue DAPI (4',6-Diamidin-2-phenylindol) channel was used for imaging of DAPI which has an excitation wavelength of 360 nm and an emission wavelength of 460 nm. 16 continuous images of each sample were processed with the corresponding softWoRx 5.5 image analysis software to generate one composite image. These images were evaluated with the help of the Volocity 6.1.1 image analysis software (Perkin Elmer). Eukaryotic nuclei were counted by measuring DAPI stained objects within a certain intensity threshold and with defined size parameters. GFP-fluorescent bacteria adherent to glass were distinguished from bacteria that co-localize with FITC staining.

5. Appendix

5.1 Comparison of *yjjQ* nucleotide sequences and deduced amino acid sequences of different *E. coli* strains

Plasmids pKEKD31 and pKERV17 encode *yjjQ* originating from *E. coli* K-12 whereas *yjjQ* encoded by the plasmids pKEKD30 and pKEKD25 originates from the UPEC strains 536 and CFT073, respectively (Table 13).

Numbers in brackets indicate the first nucleotide position in one sequence line and asterisks indicate stop codons. Divergences in the nucleotide sequence are highlighted in yellow with the aberrant nucleotide depicted in red and bold. The affected amino acid is highlighted in green.

```
      nt position  1         10         20         30         40         50         60
 aa sequence K-12  MetLeuProGlyCysCysLysAsnGlyIleValIleSerLysIleProValMetGlnAlaGly
    yjjQ K-12 (1)  ATGTTGCCAGGATGCTGCAAAAATGGAATTGTTATCAGTAAAATACCTGTTATGCAAGCAGGG
  yjjQ CFT072 (1)  ATGTTGCCAGGATGCTGCAAAAATGGAATTGTTATCAGTAAAATACCTGTTATGCAAGCAGGG
     yjjQ 536 (1)  ATGTTGCCAGGATGCTGCAAAAATGGAATTGTTATCAGTAAAATACCTGTTATGCAAGCAGGG
 aa sequence UPEC  MetLeuProGlyCysCysLysAsnGlyIleValIleSerLysIleProValMetGlnAlaGly

      nt position        70         80         90        100        110        120
 aa sequence K-12  LeuLysGluValMetArgThrHisPheProGluTyrGluIleIleSerSerAlaSerAlaGlu
   yjjQ K-12 (64)  TTAAAAGAGGTCATGAGGACTCACTTCCCTGAATATGAAATAATATCCAGCGCCTCTGCGGAG
 yjjQ CFT072 (64)  TTAAAAGAGGTCATGAGGACTCACTTCCCTGAATATGAAATAATATCCAGCGCCTCTGCGGAG
    yjjQ 536 (64)  TTAAAAGAGGTCATGAGGACTCACTTCCCTGAATATGAAATAATATCCAGCGCCTCTGCGGAG
 aa sequence UPEC  LeuLysGluValMetArgThrHisPheProGluTyrGluIleIleSerSerAlaSerAlaGlu

      nt position     130        140        150        160        170        180
 aa sequence K-12  AspLeuThrLeuLeuGlnLeuArgArgSerGlyLeuValIleAlaAspLeuAlaGlyGluSer
  yjjQ K-12 (127)  GACCTTACCTTATTACAATTACGTCGTTCCGGATTAGTCATTGCTGATTTAGCCGGTGAAAGT
yjjQ CFT072 (127)  GACCTTACCTTATTACAATTACGTCGTTCCGGATTAGTCATTGCTGATTTGGCCGGTGAAAGT
   yjjQ 536 (127)  GACCTTACCTTATTACAATTACGTCGTTCCGGATTAGTCATTGCTGATTTAGCCGGTGAAAGT
 aa sequence UPEC  AspLeuThrLeuLeuGlnLeuArgArgSerGlyLeuValIleAlaAspLeuAlaGlyGluSer

      nt position  190        200        210        220        230        240        250
 aa sequence K-12  GluAspProArgSerValCysGluHisTyrTyrSerLeuIleSerGlnTyrArgGluIleHis
  yjjQ K-12 (190)  GAAGATCCACGTTCTGTTTGTGAACATTATTATTCTTTAATCTCACAATATCGGGAAATTCAC
yjjQ CFT072 (190)  GAAGATCCACGTTCCGTTTGTGAGCATTATTACTCTTTAATCTCACAATATCGGGAAATTCAC
   yjjQ 536 (190)  GAAGATCCACGTTCCGTTTGTGAGCATTATTACTCTTTAATCTCACAATATCGGGAAATTCAC
 aa sequence UPEC  GluAspProArgSerValCysGluHisTyrTyrSerLeuIleSerGlnTyrArgGluIleHis

      nt position        260        270        280        290        300        310
 aa sequence K-12  TrpValPheMetValSerArgSerTrpTyrSerGlnAlaValGluLeuLeuMetCysProThr
  yjjQ K-12 (253)  TGGGTTTTCATGGTGTCACGCTCCTGGTATTCCCAGGCAGTAGAACTGCTCATGTGCCCTACG
yjjQ CFT072 (253)  TGGGTTTTCATGGTGTCACGCTCCTGGTATTCCCAGGCAGTAGAATTGCTCATGTGCCCTACG
   yjjQ 536 (253)  TGGGTTTTCATGGTGTCACGCTCCTGGTATTCCCAGGCAGTAGAATTGCTCATGTGCCCTACG
 aa sequence UPEC  TrpValPheMetValSerArgSerTrpTyrSerGlnAlaValGluLeuLeuMetCysProThr
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      nt position      320        330        340        350        360        370
 aa sequence K-12  AlaThrLeuLeuSerAspValGluProIleGluAsnLeuValLysThrValArgSerGlyAsn
  yjjQ K-12 (316)  GCGACGTTATTGTCTGATGTTGAACCCATTGAGAATCTGGTCAAGACCGTACGTTCCGGCAAT
yjjQ CFT072 (316)  GCGACGTTATTGTCTGATGTTGAACCCATTGAGAATCTGGTCAAGACCGTACGTTCCGGCAAT
   yjjQ 536 (316)  GCGACGTTATTGTCTGATGTTGAACCCATTGAGAATCTGGTCAAGACCGTACGTTCCGGCAAT
 aa sequence UPEC  AlaThrLeuLeuSerAspValGluProIleGluAsnLeuValLysThrValArgSerGlyAsn

      nt position   380        390        400        410        420        430        440
 aa sequence K-12  ThrHisAlaGluArgIleSerAlaMetLeuThrSerProAlaMetThrGluThrHisAspPhe
  yjjQ K-12 (379)  ACGCACGCAGAGCGTATCAGCGCCATGCTGACCTCCCCGGCAATGACTGAAACTCATGATTTT
yjjQ CFT072 (379)  ACGCACTCAGAGCGTATCAGCGCCATGCTGACCTCTCCGGCGATGACTGAAACTCATGATTTT
   yjjQ 536 (379)  ACGCACTCAGAGCGTATCAGCGCCATGCTGACCTCTCCGGCGATGACTGAAACTCATGATTTT
 aa sequence UPEC  ThrHisSerGluArgIleSerAlaMetLeuThrSerProAlaMetThrGluThrHisAspPhe

      nt position          450        460        470        480        490        500
 aa sequence K-12  SerTyrArgSerValIleLeuThrLeuSerGluArgLysValLeuArgLeuLeuGlyLysGly
  yjjQ K-12 (442)  AGCTATCGCTCCGTCATTCTCACTCTTTCAGAGCGCAAGGTACTGCGGCTATTAGGTAAAGGA
yjjQ CFT072 (442)  AGCTATCGCTCCGTCATTCTCACTCTTTCAGAGCGCAAGGTACTGCGGCTATTAGGTAAAGGA
   yjjQ 536 (442)  AGCTATCGCTCCGTCATTCTCACTCTTTCAGAGCGCAAGGTACTGCGGCTATTAGGTAAAGGA
 aa sequence UPEC  SerTyrArgSerValIleLeuThrLeuSerGluArgLysValLeuArgLeuLeuGlyLysGly

      nt position       510        520        530        540        550        560
 aa sequence K-12  TrpGlyIleAsnGlnIleAlaSerLeuLeuLysLysSerAsnLysThrIleSerAlaGlnLys
  yjjQ K-12 (505)  TGGGGCATCAACCAGATAGCTTCATTGCTTAAGAAAAGTAATAAAACTATCAGCGCCCAAAAA
yjjQ CFT072 (505)  TGGGGCATCAACCAGATAGCTTCATTGCTTAAGAAAAGTAATAAAACTATTAGCGCCCAAAAA
   yjjQ 536 (505)  TGGGGCATCAACCAGATAGCTTCATTGCTTAAGAAAAGTAATAAAACTATTAGCGCCCAAAAA
 aa sequence UPEC  TrpGlyIleAsnGlnIleAlaSerLeuLeuLysLysSerAsnLysThrIleSerAlaGlnLys

      nt position    570        580        590        600        610        620        630
 aa sequence K-12  AsnSerAlaMetArgArgLeuAlaIleHisSerAsnAlaGluMetTyrAlaTrpIleAsnSer
  yjjQ K-12 (568)  AACAGTGCGATGCGTCGACTGGCAATTCACAGCAACGCTGAAATGTATGCATGGATAAATAGC
yjjQ CFT072 (568)  AACAGTGCGATGCGTCGACTGGCAATTCACAGCAACGCTGAAATGTATGCATGGATAAATAGC
   yjjQ 536 (568)  AACAGTGCGATGCGTCGACTGGCAATTCACAGCAACGCTGAAATGTATGCATGGATAAATAGC
 aa sequence UPEC  AsnSerAlaMetArgArgLeuAlaIleHisSerAsnAlaGluMetTyrAlaTrpIleAsnSer

      nt position           640        650        660        670        680        690
 aa sequence K-12  AlaGlnGlyAlaArgGluLeuAsnLeuProSerValTyrGlyAspAlaAlaGluTrpAsnThr
  yjjQ K-12 (631)  GCGCAGGGTGCAAGAGAACTTAACTTGCCTTCTGTTTATGGAGATGCCGCAGAATGGAACACA
yjjQ CFT072 (631)  GCGCAGGGTGCAAGAGAACTTAACTTGCCTTCTGTTTATGGAGATGCCGCAGAATGGAACACA
   yjjQ 536 (631)  GCGCAGGGTGCAAGAGAACTTAACTTGCCTTCTGTTTATGGAGATGCCGCAGAATGGAACACA
 aa sequence UPEC  AlaGlnGlyAlaArgGluLeuAsnLeuProSerValTyrGlyAspAlaAlaGluTrpAsnThr

      nt position        700        710        720   726
 aa sequence K-12  AlaGluLeuArgArgGluMetSerHisSer***
  yjjQ K-12 (694)  GCCGAATTAAGAAGAGAAATGTCGCACTCATAG
yjjQ CFT072 (694)  GCCGAATTAAGAAGAGAAATGTCGCACTCATAG
   yjjQ 536 (694)  GCCGAATTAAGAAGAGAAATGTCGCACTCATAG
 aa sequence UPEC  AlaGluLeuArgArgGluMetSerHisSer***
```

One nucleotide of the *yjjQ* sequence (nt 177) differs among the UPEC strains but it has no influence on the protein level. This is also true for seven *yjjQ* nucleotides (nts 204, 213, 222,

298, 414, 420, 555) that differ between K-12 and both UPEC strains. Only the nucleotide variation at position 385 is reflected in the YjjQ protein.

5.2 Summary of the microarray data analysis

On the following pages, a schedular summary of the microarray data is given (Table 15). Loci affected by YjjQ are ordered by their genome positions in *E. coli* strain K-12 and UPEC strain CFT073, respectively. Arrows pointing to the left or right indicate the chromosomal orientation of the genes and bidirectional arrows represent intergenic regions. Probes that are aligned left or right are strain-specific and probes with justified positions matched in both bacterial strains. Genes depicted in bold are the ones which are regulated by YjjQ and loci depicted in red are regulated in both bacterial strains. Boxes around two or more genes indicate putative transcription units. Within the columns, green indicates upregulated genes, light red indicates unregulated and slightly regulated genes, and red indicates downregulated genes having a fold repression value of 5 and more.

Table 15: Summary of the microarray data analysis.

K12 probe ID	CFT073 probe ID	K12 name	CFT073 name	K12 position	CFT073 position	K12 acc. No.	CFT073 acc. No.	synonyms	K12	CFT073
1760828_s_at		cra		88,028→89,032		b0080		serR?, **fruR**, fruC, shl	-3,4	-1,9
1760840_s_at		yadE		145,081	155,286	b0130	C0159		-1,2	1,2
1765227_s_at		**panD**		146,314←146,694	156,519←156,899	b0131	C0160		-11,4	-10,4
	1767264_s_at		c0161		156,867→157,052		C0161		-1,0	1,1
1764265_s_at		yadD		147,870	158,117	b0132	C0162		-1,1	-1,1
	1762622_at								1,2	-1,2
	1767188_s_at		c0410		390,151		C0410		-1,0	-4,0
	1767640_s_at		**c0411**		391,165→392,154		C0411		1,2	-8,4
	1761753_at		c0412		393,247		C0412		1,2	-2,0
1763938_s_at		moaE		818,518	841,916	b0785	C0867	chlA5	1,1	1,0
1764111_s_at		**ybhL**		819,107→819,811	842,424→843,209	b0786	C0868		-26,8	-19,3
	1759186_s_at		**c0869**		843,141←843,272		C0869		-11,4	-2,9
1764422_s_at		ybhM		820,729	844,128	b0787	C0870		-1,5	-1,0
	1763070_at								-1,1	-1,4
1760441_s_at		ompF ↔ asnS		986,226↔986,807		-			-6,4	-1,9
1768665_s_at		gfcA↔ insAB-4 ↔ cspH gfc regulatory region		1,049,767↔1,050,182		-		ymcD	-53,1	1,0
1764937_s_at		torD		1,061,022	1,096,506	b0998	C1134		1,1	1,2
1761834_s_at		**cbpM**		1,061,773←1,062,078	1,097,494←1,097,799	b0999	C1135	yccD	-10,1	-4,9
1767087_s_at		**cbpA**		1,062,078←1,062,998	1,097,799←1,098,719	b1000	C1136		-25,3	-8,0
1759330_at		yccE		1,063,259→1,064,515		b1001			-1,1	1,1
1760217_s_at		agp		1,066,049	1,100,210	b1002	C1137		1,1	1,0
	1759895_at								-1,1	1,0
	1765428_at		c1615		1,459,716		C1615		1,1	-1,3
	1766969_s_at		**ymgD**		1,460,301←1,460,636		C1616		-1,9	-9,9
	1763277_s_at		**c1617**		1,460,640←1,460,984		C1617		-2,4	-12,0
	1761557_s_at		**c1618**		1,460,986←1,461,159		C1618		-3,2	-21,4
	1763523_s_at		c1619		1,461,903		C1619		-1,0	1,0
1765835_s_at		cysB		1,331,879	1,578,166	b1275	C1742		-1,2	1,1
1761738_s_at		**ymiA**		1,333,184→1,333,312	1,579,435→1,579,599	b4522	C1743	yciX_1	-23,3	-10,4
1764922_s_at		**yciX**		1,333,315→1,333,482	1,579,581→1,579,769	b4523	C1744	yciX_2	-28,6	-10,1
1760881_s_at		acnA		1,336,530	1,582,803	b1276	C1745		-1,2	-1,5

K12 probe ID	CFT073 probe ID	K12 name	CFT073 name	K12 position	CFT073 position	K12 acc. No.	CFT073 acc. No.	synonyms	K12	CFT073
no probe		ynaE		1,432,015		b1375			-	-
	1766831_at		c1820		1,652,469		C1820		1,2	-1,8
1760228_s_at		**uspF**		1,433,209←1,433,643	1,652,926←1,653,432	b1376	C1821	yzzL, ynaF	-6,4	-10,6
1761418_s_at		ompN		1,434,917	1,654,634	b1377	C1822	ynaG	-1,0	-1,2
1766178_s_at									-1,3	-1,1
no probe		ydgU		1,669,801		b4601			-	-
1759761_s_at		**ydgD**		1,669,984→1,670,805	1,832,655	b1598	C1990		-6,7	-4,7
1763040_s_at		**mdtI**		1,670,844←1,671,173	1,833,515←1,833,844	b1599	C1991	ydgE	-8,8	-7,3
1759134_s_at		**mdtJ**		1,671,160←1,671,525	1,833,831←1,834,196	b1600	C1992	ydgF	-31,1	-13,7
1766227_s_at		mdtJ ↔ tqsA	ydgF ↔ c1993	1,671,599↔1,671,906	1,834,270↔1,834,577	-	-		-16,3	-11,4
1761399_s_at		tqsA		1,672,971	1,835,642	b1601	C1993	ydgG	-1,6	-2,5
	1763982_s_at		motA		2,125,705		C2305		1,0	-1,2
	1760305_s_at		**flhC**		2,126,718←2,127,296		C2306		1,1	-6,0
	1764028_s_at		**c2307**		2,126,795←2,126,941		C2307		1,1	-5,0
	1762824_s_at		**flhD**		2,127,299←2,127,658		C2308		1,0	-9,8
	1760336_s_at		yecG		2,128,857		C2309		1,0	-1,3
1765972_s_at		rcnR		2,183,818	2,476,196	b2105	C2632	yohL	1,1	-1,0
1761374_s_at		rcnA		2,183,939 -> 2,184,763	2,476,589→2,477,413	b2106	C2633	yohM	-1,2	-1,0
1759340_s_at		**rcnB**		2,184,982 -> 2,185,320	2,477,452→2,477,970	b2107	C2634	yohN	-5,0	-5,2
1765584_s_at		yehA		2,185,402	2,479,086	b2108	C2635		1,1	-1,1
1759114_s_at		apbE		2,308,501	2,597,422	b2214	C2756	yojK, yojL	-1,4	1,2
	1766378_s_at		**c2757**		2,598,529→2,599,623		C2757		-29,7	-13,0
1768937_at		**ompC**		2,309,668←2,310,771	2,598,589←2,599,716	b2215	C2758	meoA, par	-36,6	-1,1
	1760091_s_at								1,1	-17,2
1762699_at		micF		2,311,106→2,311,198		b4439		stc	2,4	1,2
1762895_s_at		rcsD		2,311,510→2,314,182	2,600,455→2,603,127	b2216	C2759	yojP, yojQ, yojN	1,0	1,1
1767513_s_at		rcsB		2,314,199→2,314,849	2,603,099→2,603,794	b2217	C2760		1,0	1,1
1768567_s_at		rcsC		2,317,850	2,606,729	b2218	C2761		1,1	1,1
1769181_at		ypdI		2,492,720	2,777,081	b2376	C2912		-1,0	-1,1
	1761386_at								1,2	-1,2
	1761982_s_at		c2913		2,777,341←2,777,478		C2913		-4,1	-4,1
1766480_s_at		**yfdY**		2,493,072←2,493,314	2,777,433←2,777,675	b2377	C2914		-5,3	-7,8
1759732_s_at		lpxP		2,494,587	2,778,948	b2378	C2915	ddg	1,0	-1,1

K12 probe ID	CFT073 probe ID	K12 name	CFT073 name	K12 position	CFT073 position	K12 acc. No.	CFT073 acc. No.	synonyms	K12	CFT073
1762292_s_at		cysP		2,540,534	2,822,447	b2425	C2959		1,4	1,2
	1764785_at								1,1	1,3
1761380_s_at		**ucpA**		2,541,854←2,542,645	2,823,621←2,824,568	b2426	C2960	yfeF	-22,0	-8,7
	1759310_at								-1,4	-3,6
1761757_at		murR		2,542,774←2,543,631		b2427		yfeT	-1,0	-1,2
1760563_at		murQ		2,544,691	2,825,588	b2428	C2961	yfeU	1,1	-1,2
	1763853_at								1,2	1,3
1759305_s_at		yfiL		2,739,382	3,004,042	b2602	C3123		-1,7	-1,3
1761363_s_at		**yfiR**		2,739,897→2,740,415	3,004,572→3,005,090	b2603	C3124		-23,4	-7,8
1759798_s_at		**yfiN**		2,740,405→2,741,631	3,005,080→3,006,306	b2604	C3125		-8,2	-5,8
1764972_s_at		**yfiB**		2,741,647→2,742,129	3,006,322→3,006,804	b2605	C3126		-4,7	-5,1
1767377_s_at		rplS		2,742,552	3,007,228	b2606	C3127		1,5	-1,0
1765655_s_at		cysD		2,873,443		b2752			-1,3	-1,6
1768783_s_at		**iap**		2,874,603→2,875,640		b2753			-5,8	-3,9
1763303_at		cas2		2,876,875		b2754		ygbF	-1,2	-1,0
1760515_s_at		yqcC		2,921,806	3,198,930	b2792	C3358		-2,2	-1,1
1763312_at		**csrB**		2,922,178←2,922,537		b4408			-11,3	-5,2
1766050_s_at		syd		2,923,302	3,199,552	b2793	C3359	ydr	1,3	-1,0
1768062_s_at		npr		3,345,988		b3206		yhbK, ptsO, rpoR	-1,1	-1,2
1762003_s_at		**yrbL**		3,346,474→3,347,106		b3207			-5,9	-2,9
1760539_s_at		mtgA		3,347,831		b3208		mgt, yrbM	1,0	1,1
	1767449_s_at		yqfA		3,339,343		C3480		-1,2	-1,1
	1759347_s_at		**yqfB**		3,340,166←3,340,477		C3481		-4,0	-5,1
	1764949_at		bglA		3,341,955		C3482		-1,7	-3,0
	1759844_at		kpsS		3,518,804		C3691		1,1	-1,8
	1763650_at		**c3692**		3,520,204←3,521,199		C3692		1,0	-5,9
	1768579_at		**c3693**		3,521,205←3,522,233		C3693		1,3	-7,6
	1767211_at		**c3694**		3,522,336←3,526,136		C3694		-1,1	-6,5
	1763972_at		**c3695**		3,526,190←3,527,425		C3695		-1,0	-7,6
	1769247_at		**c3696**		3,527,441←3,527,848		C3696		1,1	-8,2
	1764875_at		**kpsT**		3,527,848←3,528,519		C3697		1,2	-6,0
	1759111_at		**kpsM**		3,528,516←3,529,292		C3698		1,1	-6,4
	1763318_at		yghD		3,531,002		C3699		1,1	1,1

K12 probe ID	CFT073 probe ID	K12 name	CFT073 name	K12 position	CFT073 position	K12 acc. No.	CFT073 acc. No.	synonyms	K12	CFT073
no probe		yhiS_2		3,651,239		G0-10683			-	-
	1764896_at		c4303		4,083,123		C4303		-1,1	1,3
1768627_s_at		slp		3,651,984→3,652,550	4,083,657→4,084,256	b3506	C4304		-4,4	-4,8
	1768080_s_at		**c4305**		4,084,297→4,084,470		C4305		-6,3	-6,2
1763257_s_at		**dctR**		3,652,706→3,653,236	4,084,409→4,084,939	b3507	C4306	yhiF	-5,1	-3,7
1762733_s_at		yhiD		3,653,925		b3508		yhhE	-3,6	-1,1
	1763313_s_at		chuS		4,086,024		C4307		1,0	1,2
1760302_s_at		yhjK		3,681,653	4,122,353	b3529	C4341		1,1	1,4
1768459_s_at									-1,3	-4,6
1760359_s_at		bcsC		3,683,723←3,687,196	4,124,423←4,127,896	b3530	C4342	yhjL	-1,3	-1,4
	1760034_at								1,2	-4,9
1766619_s_at		**bcsZ**		3,687,178←3,688,284	4,127,878←4,128,990	b3531	C4343	bcsC, yhjM	-1,3	-5,8
1760246_s_at		**bcsB**		3,688,291←3,690,630	4,128,991←4,131,417	b3532	C4344	yhjN	-1,8	-1,8
	1759665_at								-1,0	-10,0
1760799_s_at		**bcsA**		3,690,641←3,693,259	4,131,341←4,134,007	b3533	C4345	yhjP, yhjO	-1,3	-1,2
	1763161_at								1,1	-5,5
1766617_s_at		**bcsQ**		3,693,256←3,693,984	4,133,956←4,134,708	b3534	C4346	yhjQ	-8,9	-13,9
1762034_s_at									-2,8	-11,6
1766976_s_at		**yhjR**		3,694,020←3,694,208	4,134,720←4,134,908	b3535	C4347		-15,5	-12,9
1764728_s_at		yhjR ↔ bcsE	yhjR ↔ yhjS	3,694,212↔3,694,480	4,134,913↔4,135,180	-	-		-39,5	-14,3
1760595_s_at		**bcsE**		3,694,481→3,696,052	4,135,181→4,136,752	b3536	C4348	yhjS	-9,0	-7,9
1763715_s_at		**bcsF**		3,696,049→3,696,240	4,136,749→4,136,940	b3537	C4349	yhjT	-8,7	-7,5
1767568_s_at		**bcsG**		3,696,237→3,697,916	4,136,937→4,138,616	b3538	C4350	yhjU	-7,6	-6,5
1763059_s_at		ldrD		3,698,110	4,138,944	b4453	C4351		-1,1	1,3
1765253_s_at		yjbJ		4,257,260		b4045			-1,3	-1,4
1763405_s_at		**zur**		4,257,511←4,258,026		b4046		yjbK, znuR	-5,9	1,0
1760801_at		yjbL		4,258,344		b4047			1,0	1,0
1760951_s_at		eptA		4,331,970	4,892,483	b4114	C5119	yjdB	-1,4	-1,1
	1759422_at								1,0	-1,5
1764522_s_at		**adiC**		4,333,717←4,335,054	4,894,230←4,895,567	b4115	C5120	yjdD, yjdE	-7,2	-6,5
1766003_s_at		**adiY**		4,335,191←4,335,952	4,895,704←4,896,465	b4116	C5121		-39,6	-29,3
1767254_s_at		adiA		4,338,544	4,899,071	b4117	C5122		-1,5	-1,3
1760826_s_at		yjdF		4,342,952		b4121			1,0	1,1
no probe		fumB		4,343,703←4,345,349		b4122			-	-
1761781_s_at		**dcuB**		4,345,427←4,346,767		b4123		genF	-5,1	-1,9
1762536_s_at		dcuR		4,348,057		b4124		yjdG	-1,5	-1,5

K12 probe ID	CFT073 probe ID	K12 name	CFT073 name	K12 position	CFT073 position	K12 acc. No.	CFT073 acc. No.	synonyms	K12	CFT073
	1766770_s_at		blc		4,985,910		C5237		-1,2	1,1
	1765348_s_at		ampC		4,986,532←4,987,698		C5238		-1,4	-1,3
	1760339_s_at		**frdD**		4,987,728←4,988,117		C5239		-4,2	-8,1
	1759219_s_at		**frdC**		4,988,098←4,988,493		C5240		-4,4	-8,4
	1765110_s_at		**frdB**		4,988,504←4,989,238		C5241		-3,8	-8,0
	1767636_s_at		**frdA**		4,989,231←4,991,039		C5242		-3,5	-5,9
	1760368_s_at		yjeA		4,992,341		C5243		-1,3	-1,2
1767832_at		yjhR		4,533,038		b4308			-1,2	1,0
	1759876_at		c5386		5,131,079		C5386		1,4	-1,0
1759095_s_at		**nanS**		4,534,637←4,535,617	5,131,337←5,132,317	b4309	C5387	yjhS	-16,8	1,2
1763294_s_at		**nanM**		4,535,682←4,536,788	5,132,382←5,133,596	b4310	C5388	yjhT	-9,5	1,3
1761867_s_at		**nanC**		4,536,808←4,537,524	5,133,508←5,134,233	b4311	C5389	yjhA	-11,5	1,1
	1769280_at		c5390		5,135,022←5,135,429		C5390		-1,0	-1,2
1769163_s_at		**fimB**		4,538,980→4,539,582	5,135,689→5,136,291	b4312	C5391	pil	-2,7	-6,7
1760084_s_at		fimE		4,540,656	5,137,365	b4313	C5392	hyp, pilH	-1,2	-1,6
1765480_s_at		psuK/pscK		2,256,377		b2166		yeiC	-1,1	-1,1
1764331_s_at		fruA		2,257,741←2,259,432		b2167		ptsF	4,6	-1,1
1759218_s_at		**fruK**		2,259,449←2,260,387		b2168		fpk, fruF	5,6	-1,1
1759178_s_at		**fruB**		2,260,387←2,261,517		b2169		fpr, fruF	7,3	-1,1
1766196_s_at		setB		2,263,066		b2170		yeiO	1,1	1,2
1766127_s_at		yedZ		2,039,142		b1972			-1,1	-1,1
1764146_s_at		**zinT**		2,039,399→2,040,049		b1973		yodA	7,6	2,2
1767635_s_at		yodB		2,040,392		b1974			1,2	-1,1
	1767519_s_at		ydjR		1,978,845		C2141	ves	-1,0	1,3
	1766996_s_at		c2142		1,978,973→1,979,461		C2142		1,3	3,7
	1764483_s_at		**spy**		1,978,985←1,979,470		C2143		3,5	6,0
	1760113_s_at		ydjS		1,979,800		C2144	astE	-2,1	1,2

5.3 Description of putative YjjQ target loci identified by the microarray

In the following chapter a detailed description of the functions of the proteins encoded by the putative YjjQ target genes in K-12 as well as CFT073 is given in alphabetical order (except for the *bcs* locus, see chapter 1.3.2.).

adiY adiC (39.6-fold repression in K-12, 29.3-fold repression in CFT073)

The transcriptional regulator AdiY and the arginine-agmatine antiporter AdiC are involved in the arginine-dependent acid resistance in bacteria. In *E. coli*, overexpression of AdiY induces transcription of *adiA* encoding arginine decarboxylase (Stim-Herndon *et al.*, 1996). AdiA catalyzes the synthesis of agmatine from arginine. The exchange of intracellular agmatine against extracellular arginine is mediated by AdiC.

The transcriptional regulator AdiY is repressed by H-NS. When expressed, AdiY also affects expression of some genes related to the glutamate specific pathway (e.g. *dctR*) and to general acid resistance in *E. coli* (Krin et al., 2010a). The arginine decarboxylase AdiA plays a critical role in pH homeostasis. This enzyme increases the pH by removing acidic carboxyl groups and releasing CO_2 from its substrate arginine thereby generating agmatine. The antiporter AdiC maintains cell viability under extreme acid stress conditions (Iyer *et al.*, 2003, Gong *et al.*, 2003). Its activity depends on the arginine concentration and on the pH. AdiC catalyzes electrogenic transport at neutral pH but the uptake rate is much greater at low pH (Fang *et al.*, 2007). Furthermore, AdiC is involved in YdeO-induced acid resistance (Masuda & Church, 2003). AdiC expression is elevated following overexpression of the FixJ/NarL-type transcription factor EvgA in *E. coli* (Masuda & Church, 2002). The nucleoid-associated protein HU activates expression of *adiA* and *adiC* in *E. coli* resulting in the induction of arginine-dependent acid resistance (Bi *et al.*, 2009). Moreover, both genes are repressed by H-NS and activated by RcsB/GadE heterodimers (Krin et al., 2010a, Krin *et al.*, 2010b).

cbpAM (25.3-fold repression in K-12, 8.0-fold repression in CFT073)

The curved DNA-binding protein CbpA is a major nucleoid-associated protein as well as co-chaperone in bacteria (Ueguchi *et al.*, 1994). In *E. coli*, CbpA is co-expressed with the CbpA

modulator protein CbpM (Chae *et al.*, 2004). CbpA and CbpM together modulate the activity of the DnaK/DnaJ chaperone system.

Chaperone proteins mediate a wide variety of cellular processes including nascent polypeptide folding, translocation across membranes, targeting for degradation, disassembly of macromolecular aggregates, and induction of conformational changes that affect biological functions. The chaperone DnaK is the major 70-kDa heat shock protein (Hsp70) of *E. coli*. It enables cell growth under stress conditions such as heat shock and hypothermia. Hsp70 chaperones are present in all bacteria and in all eukaryotes. Their activity is regulated by co-chaperones of the Hsp40 family in an ATP-dependent manner. The Hsp40 protein DnaJ is a co-chaperone of DnaK in *E. coli* and both proteins are involved e.g. in reactivation of heat-inactivated proteins (Genevaux *et al.*, 2007, Qiu *et al.*, 2006, Kampinga & Craig, 2010).

The *cbpAM* operon is transcriptionally controlled by RpoS and Lrp (Chenoweth & Wickner, 2008). Under some conditions, CbpA acts as a functional homolog of DnaJ in *E. coli*. CbpA binds native substrates and targets them for recognition by DnaK (Chae et al., 2004). Despite the similarities between CbpA and DnaJ, CbpM targets selectively CbpA (Chenoweth *et al.*, 2007, Bird *et al.*, 2006). The binding sites of CbpM and DnaK on CbpA overlap so that DnaK and CbpM compete for binding to CbpA (Sarraf *et al.*, 2010). CbpA binds nonspecifically to DNA but with a high affinity for curved stretches (Ueguchi et al., 1994). It forms large aggregates upon DNA binding and dimerization of CbpA is required for DNA binding (Cosgriff *et al.*, 2010). CbpM is unable to bind DNA but is able to disrupt CbpA-DNA aggregates (Chintakayala & Grainger, 2011). Interaction of CbpA with CbpM inhibits both the co-chaperone activity and the DNA-binding activity of CbpA.

cra (3.4-fold repression in K-12)

The catabolite repressor/activator protein Cra is a global regulator in bacteria controlling the expression of numerous genes associated with energy metabolism. Cra guides the direction of carbon flux in *E. coli* and consequently influences the rates of utilization of various carbon sources. It primarily functions by selecting a fermentative pathway or an oxidative pathway of carbon metabolism depending on the physiological conditions (Ramseier, 1996).

Cra was initially characterized as a transcriptional repressor of the *fruBKA* operon encoding fructose-specific enzymes and was therefore called FruR (Geerse *et al.*, 1986, Chin *et al.*, 1987) (see *fruBKA*). Cra is a LacI/GalR-type transcriptional regulator and it possesses an N-terminal HTH DNA-binding domain and a C-terminal carbohydrate-binding domain. Cra mediates responses to carbon availability by activating the expression of genes encoding biosynthetic and oxidative enzymes that are normally subject to catabolite repression. In parallel, Cra represses the expression of genes encoding glycolytic enzymes that are subject to catabolite activation (Ramseier, 1996, Saier & Ramseier, 1996). This repressive effect is reversed by carbohydrate catabolites that bind to Cra and displace it from its operator sites in the target operons. Catabolite activation thus generally occurs whenever Cra represses transcription, while catabolite repression occurs whenever Cra activates transcription. The mechanism of Cra action is independent of the cAMP/CRP control system (Chin et al., 1987). In *E. coli*, Cra synthesis is increased by polyamines (Terui *et al.*, 2007) (see *mdtJI*). Furthermore, Cra activates transcription of the *csgDEFG* operon thereby inducing curli synthesis in *E. coli* (Reshamwala & Noronha, 2011) (see chapter 1.3.1).

csrB (11.3-fold repression in K-12, 5.2-fold repression in CFT073)

The pleiotropic small regulatory RNA CsrB exerts its functions by modulating the activity of the RNA-binding protein CsrA (carbon storage regulator) in bacteria. CsrA is a global regulator playing a role in carbohydrate metabolism and pathogenicity. This post-transcriptional, mostly negative regulator binds to target mRNAs and modulates their stability and translation (Timmermans & Van Melderen, 2010, Lucchetti-Miganeh *et al.*, 2008). CsrA itself activates expression of the sRNA CsrB in *E. coli* (Gudapaty *et al.*, 2001).

CsrA suppresses biofilm formation in *E. coli* (Jackson *et al.*, 2002, Wang et al., 2005). With a few exceptions, CsrA negatively regulates c-di-GMP metabolism in *E. coli* and *S. typhimurium* (Jonas *et al.*, 2008, Jonas *et al.*, 2010). Positive roles of CsrA are e.g. promoting flagellar motility in *E. coli* (Wei *et al.*, 2001, Yakhnin *et al.*, 2013) and controlling virulence factor expression in EPEC (Bhatt *et al.*, 2009). The sRNA CsrB binds to CsrA thereby building a multi-subunit ribonucleoprotein complex. CsrB competes with cellular mRNAs for CsrA binding. This sequestration of CsrA by CsrB antagonizes its activity in mediating mRNA decay (Liu *et al.*,

1997). This could explain the fact that CsrB acts as an activator of biofilm formation (Jackson et al., 2002, Weilbacher *et al.*, 2003). The FixJ/NarL-type transcription factor UvrY is a response regulator in the BarA-UvrY TCS. Activation of this TCS in *E. coli* leads to an overproduction of CsrB (Suzuki *et al.*, 2002).

dctR (5.1-fold repression in K-12)

The FixJ/NarL-type transcription factor DctR may act as a negative transcriptional regulator of *dctA* encoding a transporter for C_4-dicarboxylates in *E. coli* (Boogerd *et al.*, 1998). It is implicated to play a role in glutamate-dependent acid resistance and in YdeO-induced acid resistance in *E. coli* (Masuda & Church, 2003). DctR-regulated gene products protect *E. coli* against the pH-dependent toxic effects of lactic, succinic, and formic acids (Mates *et al.*, 2007). DctR is encoded within an acid fitness island together with the FixJ/NarL-type transcription factor GadE. The *dctR* gene is repressed by H-NS and activated by AdiY (see *adiY adiC*) as well as RcsB/GadE heterodimers (Krin et al., 2010a, Krin et al., 2010b). DctR expression in *E. coli* is elevated following overexpression of EvgA, another FixJ/NarL-type transcription factor (Masuda & Church, 2002).

The attachment of EHEC to intestinal epithelial cells depends on the presence of the locus of enterocyte effacement (LEE) region. This locus includes genes encoding a type 3 secretion system and adhesion factors that play principal roles in bacterial adherence to host cells. DctR negatively regulates adherence and type 3 secretion in EHEC. It is presumed to serve as a negative regulator of LEE-encoded gene expression at the RNA level (Tatsuno *et al.*, 2003).

dcuB (5.1-fold repression in K-12)

DcuB is an antiporter mediating uptake of C_4-dicarboxylates, e.g. fumarate, L-malate or aspartate, against succinate. C_4-dicarboxylates play an important role in carbon metabolism of *E. coli* (Janausch *et al.*, 2002). L-Malate and aspartate are converted to fumarate and are then metabolized by fumarate respiration. The product succinate is not further catabolized in anaerobic growth and excreted by DcuB (Kleefeld *et al.*, 2009).

Expression of *dcuB* depends on FNR (fumarate nitrate reductase regulator) which activates expression of genes of anaerobic respiration, and on cAMP/CRP (Golby *et al.*, 1998). In addition,

dcuB expression requires transcriptional activation by the DcuSR TCS (Zientz *et al.*, 1998, Golby *et al.*, 1999). The sensor kinase DcuS is activated by phosphorylation in the presence of C_4-dicarboxylates. Subsequently the phosphate group is transferred to DcuR. This response regulator activates expression of target genes including the fumarate reductase genes (*frdABCD*) and *dcuB*. Being both, a C_4-dicarboxylate carrier and a cosensor for DcuS, DcuB is a bifunctional protein in *E. coli* with transport as well as regulatory functions (Kleefeld et al., 2009).

fimB (6.7-fold repression in CFT073)

The FimB recombinase is the major type 1 fimbriae (or type 1 pili) inversion protein in *E. coli* that acts as a switch regulating fimbriation. Type 1 fimbriae are the most common fimbrial adhesins produced by many UPEC and commensal *E. coli* isolates. They play a fundamental role in adhesion to mucosal surfaces, colonization of the urinary tract, and invasion into bladder epithelial cells (Bahrani-Mougeot *et al.*, 2002, Eto et al., 2007). For instance, the FimH type 1 fimbrial adhesin of UPEC mediates adhesion to mannose residues on urothelial host cells (Chen *et al.*, 2009).

Expression of type 1 fimbriae in *E. coli* is under phase-variable control. The orientation of an invertible DNA segment in the chromosome, the *fim* switch (*fimS*), determines the state of fimbriation. The *fimB* gene encodes a protein showing homology to members of the integrase family of site-specific recombinases (Dorman & Higgins, 1987). FimB modulates transcription of the fimbrial subunit gene *fimA* by repositioning of *fimS* harboring the *fimA* promoter (*fimAp*). FimB binds to inverted repeats flanking the invertible segment thereby switching the system from the ON to the OFF phase and back again at equal rates (McClain *et al.*, 1991).

Expression of *fimB* is dependent on growth media, temperature, pH as well as salt concentration (Gally *et al.*, 1993, Olsen *et al.*, 1998, Schwan et al., 2002). In *E. coli*, inversion requires the presence of at least one of two redundant accessory proteins, Lrp or IHF (Dove & Dorman, 1996, Corcoran & Dorman, 2009). The LysR-type transcription factor LrhA represses structural as well as regulatory type 1 fimbrial genes (Blumer *et al.*, 2005, Lehnen *et al.*, 2002). Expression of *fimB* is also repressed by H-NS in *E. coli* (O'Gara & Dorman, 2000) and by the osmoregulatory response regulator OmpR in UPEC (Schwan et al., 2002). The FixJ/NarL-type

transcription factor RcsB promotes fimbriation in *E. coli* by affecting the orientation of the invertible element through the control of *fimB* transcription (Schwan et al., 2007). Moreover, expression of *fimB*, similar to the adjacent *nanCMS* operon, is modulated by NanR (see *nanCMS*). The *fimB* gene is likewise repressed by NanR, but in this case repression is dependent on the presence of sialic acid (El-Labany *et al.*, 2003, Sohanpal *et al.*, 2004). Constitutive expression of type 1 fimbriae by phase-locked ON variants of *E. coli* K-12 and UPEC strain CFT073 leads to a decrease in chemotaxis and swimming motility (Lane *et al.*, 2007b).

flhDC (9.8-fold repression in CFT073)

The *flhDC* locus encodes the master regulator of flagellar motility in bacteria. *E. coli* is a peritrichously flagellated bacterium with five to ten asymmetrically distributed flagella protruding from its surface (Ping, 2010). Flagella are fundamental motility structures that play important roles in bacterial behavior and pathogenesis. For instance, in UPEC they facilitate the ascension from the bladder into the kidneys (Schwan, 2008, Lane *et al.*, 2007a).

In *E. coli* and *S. typhimurium*, the genes of the regulon coordinating flagella synthesis are divided into three hierarchical subsets: class I (early), class II (middle), and class III (late) (Kutsukake *et al.*, 1990). More than 50 genes belong to this vast regulon, most of them being organized in operons. Their expression occurs as a transcriptional cascade and is therefore temporally regulated (Chilcott & Hughes, 2000). Class I is represented by only two genes forming the *flhDC* operon. The class I promoter is a crucial point at which numerous regulatory signals have input on the decision whether to initiate or prevent flagella biosynthesis. Similar to the *csgD* promoter, many global transcriptional regulators have been implicated in positively and negatively regulating its expression (Soutourina & Bertin, 2003). For instance, expression of *flhDC* in *E. coli* and *S. typhimurium* is sensitive to environmental and cell state sensors like cAMP/CRP (Yokota & Gots, 1970, Soutourina *et al.*, 1999). Moreover, the 5' UTR of *flhDC* mRNA is bound by at least five sRNAs, one of them activating (Thomason et al., 2012) and the other four repressing *flhDC* expression (De Lay & Gottesman, 2012). Despite of their counteractive properties, *csgD* and *flhDC* mRNAs have partially overlapping sRNA regulator repertoires. Base-pairing of the sRNAs OmrA and OmrB to both mRNAs upstream the RBS interferes with translational initiation. In contrast, the cAMP/CRP-controlled sRNA McaS promotes flagella

synthesis but suppresses curli synthesis (Chambers & Sauer, 2013, Boehm & Vogel, 2012, Van Puyvelde et al., 2013) (see chapter 1.3.4).

The master regulator of flagellar motility is a heterohexameric protein consisting of four FlhD protomers and two zinc-binding FlhC protomers (Wang *et al.*, 2006). $FlhD_4C_2$ is the transcriptional activator for class II genes encoding proteins involved in the formation of the flagellar hook and basal body. It interacts with the α-CTD of RNA polymerase thereby activating transcription from class II promoters (Liu *et al.*, 1995). In addition, the alternative sigma factor FliA (σ^{28}) (Ohnishi *et al.*, 1990) and the anti-σ^{28} factor FlgM (Gillen & Hughes, 1991) are class II proteins in *S. typhimurium*. Class I and class II promoters are activated by σ^{70}-RNA polymerase whereas transcription of class III promoters is dependent on σ^{28}-RNA polymerase (Liu & Matsumura, 1995). σ^{70} is the housekeeping sigma factor of RNA polymerase which mediates most transcription events, including those of all essential genes in *E. coli*. Class III genes encode proteins involved in flagellar filament and cap formation as well as chemotaxis proteins (Chilcott & Hughes, 2000). The DNA-binding protein FliZ, encoded in a class II operon together with FliA, interferes with RpoS (σ^{38}) activity in *E. coli* thereby inhibiting curli synthesis (Pesavento et al., 2008). In this way expression of the two alternative sigma factors RpoS and FliA is reciprocally regulated (Pesavento & Hengge, 2012).

H-NS is necessary for the biogenesis as well as motor function of flagella in *E. coli* (Bertin *et al.*, 1994). The *ycgR* gene encoding a c-di-GMP-binding PilZ domain protein belongs to class III of the flagellar regulon and is associated with H-NS-dependent motility (Ko & Park, 2000b). The LysR-type transcription factor HdfR binds to the *flhDC* promoter region and negatively regulates its transcription. HdfR expression is in turn repressed by H-NS. Thus, activation of the *flhDC* operon by H-NS is mediated indirectly through the repression of the negative regulator HdfR (Ko & Park, 2000a). Another LysR-type transcription factor, LrhA, suppresses the *flhDC* operon and thereby flagellar motility as well (Lehnen et al., 2002). Beyond that, LrhA is known to repress RpoS stability, type 1 fimbriae synthesis, and biofilm formation (Blumer et al., 2005).

The DnaK/DnaJ Hsp chaperone system is required for flagella synthesis in *E. coli* (Shi *et al.*, 1992) (see *cbpAM*). The DnaK/DnaJ machinery is involved in activating the $FlhD_4C_2$ complex into a transcriptional regulator in *S. typhimurium* (Takaya *et al.*, 2006). The enzyme fumarate

reductase (FRD) modulates flagellar motility in *E. coli* dependent on its substrate fumarate (Cohen-Ben-Lulu *et al.*, 2008) (see *frdABCD*). The global regulatory RNA-binding protein CsrA promotes flagellar motility in *E. coli* by binding to the *flhDC* transcript thereby enhancing its stability (Wei et al., 2001, Yakhnin et al., 2013) (see *csrB*). The FixJ/NarL-type transcription factor MatA, the activator of mat fimbriae expression, represses *flhDC* transcription in *E. coli* thereby promoting bacterial adhesion (Lehti *et al.*, 2012a). In Addition, *flhDC* expression may be modulated by IHF (Shin & Park, 1995). Flagella expression in *E. coli* is negatively regulated by the osmoregulatory response regulator OmpR. This repression is mediated by a direct interaction of phosphorylated OmpR with the *flhDC* promoter (Shin & Park, 1995). Moreover, the RcsCDB TCS negatively regulates expression of the *flhDC* operon in *E. coli* by binding of the RcsB/RcsA heterodimer to its promoter (Francez-Charlot et al., 2003).

frdABCD (5.9-fold repression in CFT073)

In *E. coli*, the fumarate reductase enzyme complex (FRD) catalyzes the reduction of fumarate to succinate in the final step of anaerobic respiration. The C_4-dicarboxylate fumarate therefore represents the terminal electron acceptor for oxidative phosphorylation. Fumarate respiration results in the generation of a proton potential and drives ATP synthesis and growth of bacteria (Kleefeld et al., 2009).

FRD of *E. coli* is a membrane-bound complex composed of four polypeptides. FrdA and FrdB provide the catalytic component whereas FrdC and FrdD account for the membrane anchor (Cecchini *et al.*, 2002). The *frd* genes comprise an operon and FNR activates its transcription in response to anaerobiosis. The *frdABCD* operon is coordinately regulated by the presence of alternate electron acceptors since its transcription is affected by oxygen, nitrate, and fumarate (Jones & Gunsalus, 1985, Jones & Gunsalus, 1987). Expression of the *frd* genes in *E. coli* is also controlled by the DcuSR TCS (Zientz et al., 1998) (see *dcuB*).

Fumarate modulates flagellar motility in *E. coli* by altering the direction of flagellar rotation (Prasad *et al.*, 1998, Montrone *et al.*, 1998). The structural element responsible for this action is the switch of the flagellar motor. The effects of fumarate on motility are transmitted via a reversible interaction between FRD and the flagellar switch in *E. coli* (see *flhDC*). FRD, an

enzyme known to be active primarily under anaerobic conditions therefore has important functions under aerobic conditions as well (Cohen-Ben-Lulu et al., 2008).

fruBKA (7.3-fold activation in K-12)

The permease FruAB is part of the fructose-specific phosphotransferase system (PTS). FruAB is located in the inner membrane of the bacterial cell wall where it mediates uptake of fructose (Postma *et al.*, 1993). The *fruK* gene encodes 1-phosphofructokinase, an enzyme that is essential for the utilization of fructose as carbon source. During transport, fructose is phosphorylated by the PTS in a phosphoenolpyruvate (PEP)-dependent reaction to fructose-1-phosphate. 1-Phosphofructokinase catalyzes the ATP-dependent phosphorylation of fructose-1-phosphate to fructose-1,6-bisphosphate, a central metabolite during glycolysis in most bacterial cells (Buschmeier *et al.*, 1985). The *fruBKA* operon is repressed by Cra (Ramseier et al., 1993) and activated by the cAMP/CRP complex (Shimada *et al.*, 2011b).

gfc (53.1-fold repression in K-12)

The *gfc* locus is required for polysaccharide secretion and assembly of the bacterial capsule. Capsules are protective surface-enveloping structures that represent virulence factors in bacteria. The *gfc* locus is absent from the genome of UPEC strain CFT073. *E. coli* K-12 contains an intact *gfc* locus but does not express it because of the presence of an IS*1* insertion element in its promoter region (Peleg *et al.*, 2005, Shifrin *et al.*, 2008).

E. coli produces two serotype-specific surface polysaccharides: the LPS O antigen and the capsular polysaccharide K antigen. Polysaccharide capsules of *E. coli* are classified into four groups (Whitfield & Roberts, 1999, Whitfield, 2006). The *gfcABCDE-etp-etk* operon mediates assembly of the group 4 capsule. *E. coli* contains paralogs of the *gfcABCD* genes called *yjbEFGH* (Sathiyamoorthy *et al.*, 2011). Overexpression of *yjbEFGH* altered colony morphology and led to increased production of exopolysaccharide. The expression of this operon is controlled by RcsB/RcsA heterodimers (Ferrières et al., 2007). Moreover, *yjbEFGH* is induced by osmotic stress and suppressed by RpoS (Ionescu & Belkin, 2009).

iap (5.8-fold repression in K-12)

iap encodes a proteolytic enzyme with aminopeptidase activity catalyzing the conversion of alkaline phosphatase isozyme 1 into isozymes 2 and finally 3 in *E. coli* (Ishino *et al.*, 1987, Nakata *et al.*, 1978). Alkaline phosphatase is an example for an enzyme whose catalytic activity is dependent on a zinc cofactor. It mediates cleavage of a phosphomonoester in the presence of H_2O to alcohol and inorganic phosphate.

kpsMT (6.4-fold repression in CFT073)

The *kpsMT* operon is involved in capsule synthesis in *E. coli*. Together the KpsM and KpsT proteins constitute an ABC-transporter catalyzing the export of group 2 capsular polysaccharides (Reizer *et al.*, 1992).

The *kps* gene cluster in *E. coli* comprises three functional regions (Boulnois *et al.*, 1987). The whole cluster is absent in K-12 laboratory strains and commensal isolates, but UPEC strains CFT073 and 536 express all regions (Buckles *et al.*, 2009). Region three accommodates the *kpsMT* operon which encodes two proteins for the energy-dependent translocation of capsular polysaccharides to the cell surface (Kröncke *et al.*, 1990). It is best studied in the *E. coli* serotypes K1 and K5 which therefore represent model systems for group 2 capsule synthesis (Whitfield, 2006). KpsM is an integral membrane protein and functions as a carrier mediating transport of polysaccharides across the inner membrane. KpsT is a peripheral inner membrane protein that harbors conserved ATP binding fold sequences. KpsT couples ATP hydrolysis to the early stages of the transport process (Smith *et al.*, 1990, Pavelka *et al.*, 1991). KpsMT may recognize the nascent polysaccharide and coordinate synthesis with translocation (Bliss & Silver, 1996).

mdtJI (31.1-fold repression in K-12, 13.7-fold repression in CFT073)

The MdtJI complex belongs to the small multidrug resistance (SMR) family of exporters. In prokaryotes spermidine is one of the prevailing polyamines (Igarashi & Kashiwagi, 2010). MdtJI catalyzes the excretion of excess spermidine in *E. coli* thereby enhancing cell viability (Higashi *et al.*, 2008).

Polyamines are essential for normal cell growth in prokaryotes and eukaryotes. In both organisms, cellular polyamine levels rise concomitantly with the proliferation rate (Igarashi & Kashiwagi, 2010). Polyamines interact with acidic substances like nucleic acids. In *E. coli*, about 90% of the spermidine content is associated with RNA (Miyamoto *et al.*, 1993). Spermidine itself increases the level of *mdtJI* mRNA. In this way this polyamine contributes to the relief from toxicity by its own overaccumulation (Higashi et al., 2008). Polyamines promote protein synthesis in several ways. In *E. coli*, they increase the synthesis of e.g. the nucleoid-associated protein and global repressor H-NS, adenylate cyclase, the global transcriptional regulator Cra, and the general stress sigma factor RpoS at the level of translation (Yoshida *et al.*, 2004, Igarashi & Kashiwagi, 2006, Terui et al., 2007). Since these four proteins are directly involved in transcription events, the synthesis of many mRNAs is regulated indirectly by polyamines.

nanCMS (11.5-fold repression in K-12)

The Nan system is involved in N-acetylneuraminic acid (Neu5Ac) metabolism in *E. coli*. The *nanC* gene encodes an outer membrane porin responsible for Neu5Ac uptake (Giri *et al.*, 2012). The *nanM* gene encodes an epimerase for conversion of Neu5Ac isomers (Severi *et al.*, 2008). The *nanS* gene encodes an esterase catalyzing deacetylation of Neu5Ac (Rangarajan *et al.*, 2011).

Neu5Ac is the most common sialic acid, a structurally complex group of nine-carbon monosaccharides with unique physiochemical properties (Vimr *et al.*, 2004). Expression of sialic acid catabolic operons in *E. coli* is repressed by NanR in the absence of the inducer sialic acid (Kalivoda *et al.*, 2003). Furthermore, *nanCMS* expression may depend on cAMP/CRP (Condemine *et al.*, 2005). Polymers of sialic acid are primary constituents of capsules thereby constituting virulence factors of certain pathogenic bacteria. Sialic acid catabolism regulated by NanR plays an important role for biofilm formation of UPEC as the capsule is a key element of the IBC matrix (Anderson *et al.*, 2010).

Sialic acids cap the terminal positions of glycan chains on mammalian cell surface proteins and lipids generating glycoconjugates with biologically diverse features. Bacteria obtain sialic acids either by *de novo* biosynthesis or by acquisition from the environment, i.e. by scavenging directly from the host. Many bacterial pathogens decorate their surfaces with sialic acid to interact specifically with different host tissues. Incorporation of sialic acid into the bacterial cell

wall further allows pathogens to disguise themselves as host thereby circumventing the immune response (Severi *et al.*, 2007, Vimr, 2013).

When the general porins OmpC and OmpF are absent (see *ompC*), the NanC channel is the only entry route for Neu5Ac into bacterial cells. Expression of *nanC* is activated by the EnvZ-OmpR and the CpxAR TCSs (Condemine et al., 2005). In *E. coli*, *nanC* expression and expression of the adjacent divergently transcribed *fimB* gene is coordinately controlled by shared regulatory elements. This intricate regulation may be the reason for the exceptional length of the *nanC-fimB* intergenic region (Sohanpal *et al.*, 2007, Condemine et al., 2005).

ompC (36.6-fold repression in K-12, 17.2-fold repression in CFT073)

OmpC is one of the major outer membrane porins in bacteria. In *E. coli*, OmpC forms a transmembrane β-barrel that assembles as homotrimer or heterotrimer with its paralog OmpF. These aqueous channels allow passive diffusion of hydrophilic molecules including nutrients across the outer membrane (Nikaido, 1994, Nikaido, 2003).

Due to its small pore size, OmpC may restrict the influx of toxins and antibiotics (Delcour, 2009, Pages *et al.*, 2008). Although known to be involved in rather nonspecific uptake processes, OmpC has been implicated in protein export (Prehna *et al.*, 2012). Moreover, OmpC plays a role in arginine- and lysine-dependent acid resistance (Bekhit *et al.*, 2011). Polyamines can inhibit porin function thereby decreasing outer membrane permeability. This mechanism may protect bacteria from acid stress (Iyer & Delcour, 1997, Yohannes *et al.*, 2005).

Expression of OmpC and OmpF in *E. coli* is reciprocally regulated by medium osmolarity. For that reason these proteins are referred to as osmoporins. The response regulator OmpR is able to bind to the promoter regions of both osmoporin genes. At high medium osmolarity, phosphorylated OmpR represses *ompF* expression but activates *ompC* expression (Yoshida et al., 2006, Lan & Igo, 1998). Activation of the CpxAR TCS likewise results in a decrease in *ompF* expression and an increase in *ompC* expression (Batchelor *et al.*, 2005). Thus, a complex interplay of the EnvZ-OmpR TCS and the CpxAR TCS occurs on the *ompC* promoter, similar to the one found on the *csgD* promoter (see chapter 1.3.1).

The integration host factor (IHF) represses *ompC* expression in two ways, indirectly by influencing the expression of OmpR and directly by binding to the *ompC* regulatory region

thereby inhibiting transcription (Huang *et al.*, 1990). Moreover, the leucine-responsive regulatory protein (Lrp) (Ferrario *et al.*, 1995), the sRNA MicC (Chen *et al.*, 2004), and H-NS (Pratt *et al.*, 1996) repress *ompC* transcription.

panD (11.4-fold repression in K-12, 10.4-fold repression in CFT073)

The *panD* gene encodes L-Aspartate-α-decarboxylase in *E. coli*. The native enzyme catalyzes the cleavage of aspartate to β-alanine and CO_2 (Williamson & Brown, 1979, Cronan, 1980). β-Alanine is required for the generation of an essential precursor for Coenzyme A and acyl-carrier protein biosynthesis (Ramjee *et al.*, 1997, Kennedy & Kealey, 2004). In *E. coli*, PanD is activated by the putative N-acetyltransferase PanZ (Nozaki *et al.*, 2012).

rcnB (5.0-fold repression in K-12, 5.2-fold repression in CFT073)

The periplasmic protein RcnB is involved in cobalt and nickel resistance in bacteria. Cobalt and nickel are essential trace elements in prokaryotes and eukaryotes because they serve as cofactors of several enzymes (Kobayashi & Shimizu, 1999, Kaluarachchi *et al.*, 2010).

In *E. coli*, expression of RcnB is activated by cobalt and nickel and is negatively regulated by the metalloregulator RcnR. Cobalt and nickel are toxic in excess causing growth arrest and cell death. The only identified mechanism of resistance to these substances in *E. coli* is executed by the RcnA efflux pump (Rodrigue *et al.*, 2005). RcnB is coexpressed with RcnA. Since RcnB does not bind cobalt or nickel directly, its role is most likely to modulate the efflux of nickel mediated by RcnA. In this way RcnB is critical for maintaining a proper cobalt and nickel homeostasis in *E. coli* (Blériot *et al.*, 2011). Nickel was reported to promote biofilm formation in *E. coli* K-12 as subinhibitory concentrations of nickel activate the expression of the *csg* genes encoding curli (Perrin *et al.*, 2009).

spy (6.0-fold activation in CFT073)

The spheroplast protein Y (Spy) is a homodimeric chaperone that acts independently of energy cofactors such as ATP (Quan *et al.*, 2011). Moreover, Spy is a potent inhibitor of curli formation in *E. coli* (Evans *et al.*, 2011). It was identified as a periplasmic protein whose expression is induced by spheroplast formation in *E. coli* (Hagenmaier *et al.*, 1997). Expression of *spy* is stimulated by copper (Yamamoto & Ishihama, 2005) and zinc (Yamamoto *et al.*, 2008) mediated

by the TCSs CpxAR (Raivio *et al.*, 2000) and BaeSR (Raffa & Raivio, 2002), respectively. Furthermore, Spy is a putative member of the RcsCDB TCS (Hagiwara *et al.*, 2003). During the periplasmic passage of the curli subunit protein CsgA, Spy prevents premature intracellular polymerization of amyloid fibers by maintaining CsgA in a soluble form. Overexpression of Spy decreases curli formation which is reflected in reduced Congo Red binding (Evans et al., 2011).

ucpA (22.0-fold repression in K-12, 8.7-fold repression in CFT073)

UcpA ("upstream of *cysP*") is a putative oxidoreductase that is involved in furan resistance in *E. coli* (Wang *et al.*, 2012). It shows significant sequence homology with members of the short-chain dehydrogenases/reductases (SDR) family (Sirko et al., 1997).

Furans such as furfural inhibit growth of biocatalysts like ethanologenic *E. coli*. Furan addition to *E. coli* fermentations results in an initial period of slow growth during which furans are reduced to less toxic alcohols. NADH is abundant during fermentation as electron donor (Wang et al., 2012). In an effort to identify additional NADH-dependent furfural reductases in ethanologenic *E. coli*, *ucpA* was found to be induced in the presence of furfural. Expression of *ucpA* confers furan tolerance although it does not directly metabolize furfural using NADH (Wang et al., 2012). The potential binding sites for three global regulatory proteins were identified in the *ucpA* promoter region. CRP and Cra are required for full activation of *ucpA* transcription while IHF negatively affects the expression of *ucpA* (Sirko et al., 1997).

uspF (6.4-fold repression in K-12, 10.6-fold repression in CFT073)

The universal stress protein UspF is an ATP-binding protein in *E. coli* (Saveanu *et al.*, 2002). Expression of UspF is increased under carbon-limited conditions (Raman *et al.*, 2005). It confers slight resistance against oxidative stress and streptonigrin, an antibiotic whose toxicity is dependent on intracellular iron concentrations. Deletion of *uspF* results in reduced fimbriae-dependent adhesion and enhanced motility (Nachin *et al.*, 2005). This effect of UspF on fimbriation may result from different dimerization states of the protein (Nachin *et al.*, 2008).

yfiRNB (23.4-fold repression in K-12, 7.8-fold repression in CFT073)

The conserved Yfi system represents a signaling module that regulates c-di-GMP levels of bacteria in response to so far unknown environmental signals. YfiN is an active membrane-

integral DGC whose activity is repressed by YfiR, a periplasmic protein, and stimulated by the outer-membrane lipoprotein YfiB (Malone *et al.*, 2010, Malone *et al.*, 2012).

The gram-negative pathogen *Pseudomonas aeruginosa* is responsible for chronic airway infections occurring in cystic fibrosis. During long-term lung colonization, *P. aeruginosa* adapts by the formation of slow-growing persistent isolates termed small colony variants (SCVs). SCVs are strongly linked to c-di-GMP and are characterized by e.g. enhanced exopolysaccharide production (Malone et al., 2010, Malone et al., 2012). The *yfiRNB* locus was found to be related to the SCV morphotype in *P. aeruginosa*. The primary targets of c-di-GMP produced by YfiN are two exopolysaccharide systems. Up-regulation of these systems confers resistance against phagocytosis. The Yfi system constitutes an important regulatory system involved in *P. aeruginosa* biofilm formation and *in vivo* persistence (Malone et al., 2010). The mechanism of action of this system is a subject of current research (Malone et al., 2012). Moreover, the Yfi system decreases early biofilm formation and motility in *E. coli* (Sanchez-Torres *et al.*, 2011) and functions as positive regulator of biofilm formation and type 3 fimbriae expression in *Klebsiella pneumoniae* (Wilksch *et al.*, 2011).

zinT (7.6-fold activation in K-12)

ZinT is a zinc- and cadmium-binding protein involved in maintaining metal homeostasis. Expression of *zinT* is induced during zinc shortage. ZinT possesses a high affinity binding site that can accommodate preferentially Zn^{2+} or alternatively Cd^{2+} (Graham et al., 2009). The metalloregulatory transcription factor Zur is a repressor of *zinT* transcription. ZinT contributes to the ZnuA-mediated recruitment of zinc but is subordinated to ZnuA (see *zur*). Unbound ZinT can be secreted to the extracellular space where it might sequester environmental zinc (Gabbianelli *et al.*, 2011). Mutation of *zinT* leads to the suppression of biofilm formation as zinc deficiency inhibits curli expression in EHEC. Furthermore, *zinT* mutants become elongated and assume a filamentous form. Therefore, ZinT is required for curli-mediated attachment of EHEC to host cells and for maintaining the normal shape of EHEC cells under zinc-depleted conditions (Lim *et al.*, 2011).

zur (5.9-fold repression in K-12)

The metalloregulatory transcription factor Zur controls zinc uptake in *E. coli*. Zinc is indispensable for all living cells because it serves as a structural or catalytic cofactor in a large number of proteins such as RNA polymerase (King *et al.*, 2004), ribosomal proteins (Hensley *et al.*, 2011), chaperone proteins (Kim *et al.*, 2001), and zinc finger proteins (Wagner *et al.*, 2011). Putative zinc-binding proteins account or about 5% of the total proteome in *E. coli* (Andreini *et al.*, 2006).

The ATP-binding cassette (ABC)-transporter ZnuABC constitutes a high-affinity transport system for Zn^{2+}. Zur dimers repress the *znu* gene cluster by direct binding to the *znu* operator thereby leading to a cessation of Zn^{2+} import. Zur occupies its binding site only in the presence of zinc or other divalent metal cations like cobalt. When *E. coli* suffers from zinc deprivation, derepression of the ZnuABC uptake system induces zinc acquisition (Patzer & Hantke, 1998, Patzer & Hantke, 2000). ZnuABC affects virulence-associated phenotypes of UPEC strain CFT073 such as biofilm formation and swimming motility (Gunasekera *et al.*, 2009) and it imparts a competitive advantage during UTI in the murine model (Sabri *et al.*, 2009).

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Abbreviations

ABC-transporter	ATP-binding cassette-transporter
APEC	avian pathogenic *E. coli*
ATP	adenosine triphosphate
BACTH	bacterial adenylate cyclase two-hybrid system
bdar	brown, dry and rough (morphotype)
cAMP	3',5'-cyclic adenosine monophosphate
c-di-GMP	3',5'-cyclic dimeric guanosine monophosphate
CRP	cAMP receptor protein
DGC	diguanylate cyclase
DMSO	dimethylsulfoxide
ECM	extracellular matrix
eDNA	extracellular desoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohaemorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
ExPEC	extraintestinal pathogenic *E. coli*
FBS	fetal bovine serum
FRT (site)	Flp recombinase target site
GFP	green fluorescent protein
GPA	gentamicin protection assay
HR	homology region
HTH	helix-turn-helix
IBC	intracellular bacterial community
IM	inner membrane of the bacterial cell wall
IPTG	isopropyl-β-D-thiogalactopyranoside
LEE	locus of enterocyte effacement
LPS	lipopolysaccharide
MOI	multiplicity of infection
MU	Miller unit
Neu5Ac	N-acetylneuraminic acid (sialic acid)
nm	nanometer
NMEC	neonatal meningitis-causing *E. coli*
nSD	native Shine-Dalgarno sequence
nt	nucleotide

OD	optical density
OM	outer membrane of the bacterial cell wall
ONPG	2-nitrophenyl β-D-galactopyranoside
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pdar	pink, dry and rough (morphotype)
PDE	phosphodiesterase
PFU	plaque forming unit
PGA	poly-β-1,6-N-acetyl-D-glucosamine
RBS	ribosome binding site
rdar	red, dry and rough (morphotype)
saw	smooth and white (morphotype)
spp.	species pluralis
sRNA	small regulatory ribonucleic acid
TCS	two-component system
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection
UTR	untranslated region
WT	wild-type
X-gal	5-brom-4-chlor-3-indoxyl-β-D-galactopyranoside
εSD	epsilon Shine-Dalgarno sequence

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Erklärung

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Köln, 16. Juli 2013 _____

Katrin Dreck