Analysis of the interaction of the LuxR-type transcription factors BglJ and RcsB and antagonism of H-NS mediated silencing in *Escherichia coli*

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Da simmer dabei, dat is prima, VIVA COLONIA!

Contents

Abbreviations

BCA	bicinchoninic acid
bp	base pair
BTB	bromothymol blue plates
CRP	catabolite gene activator protein
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
HABA	4-hydroxy azobenzene-2-carboxylic acid
H-NS	histone-like nucleoid structuring protein
IgG	immunoglobulinG
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KDa	kilo dalton
KCl	potassium chloride
LB	Luria Bertani
NaCl	sodium chloride
OD	optical density
ONPG	o-nitrophenyl- β-D-galactopyranoside
PCR	polymerase chain reaction
PMSF	phenylmethyl sulphonyl fluoride
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCS	two-component system
wt	wild type

I. Zusammenfassung

Das nukleoid-strukturierende Protein H-NS ist eines der häufigsten DNA bindenden Proteine in *Enterobacteriaceae*. H-NS spielt eine Schlüsselrolle in der Organisation des bakteriellen Chromosoms und in der Regulation von Genaktivität. Als Repressor der Transkription reguliert H-NS in *Escherichia coli* die Aktivität von etwa 5 % des Genoms. Dabei reguliert es Genexpression in Antwort auf verschiedene Umweltsignale. H-NS wirkt dabei als globaler Repressor, indem es an spezifische DNA-Sequenzen bindet, woraufhin sich stabile Nukleoproteinkomplexe und H-NS-DNA-H-NS-Brücken ausbilden, welche die Transkriptionsinitiation verhindern können. Der H-NS-vermittelten Repression kann durch spezifische Transkriptionsfaktoren entgegengewirkt werden. Dieses Prinzip wurde bereits für mehrere H-NS-reprimierte Genloci gezeigt, z. B. für das *bgl*-Operon in *E. coli*, das als modellhaft für den Mechanismus der Repression durch H-NS gilt. Am bgl-Operon kann der LuxR-Typ-Transkriptionsfaktor BglJ die Repression durch H-NS aufheben, wobei der Antirepressoreffekt von BglJ abhängig ist von RcsB. RcsB ist der Antwortregulator des Rcs-Signaltransduktionssystems, das durch Membranstress aktiviert wird und zahlreiche zelluläre Prozesse in *Enterobacteriaceae* reguliert.

BglJ ist ein Trasnkriptionsfaktor vom LuxR-Typ und ist gemeinsam mit YjjQ in einem Operon kodiert. Frühere Analysen in einem *Two-Hybrid*-System ließen darauf schließen, dass RcsB sowohl mit BglJ als auch mit YjjQ interagieren kann. In der vorliegenden Studie wurde die Interaktion von RcsB mit BglJ und YjjQ mittels Co-Immunopräzipitation-Experimenten untersucht, die zeigten, dass RcsB eindeutig mit BglJ und weniger eindeutig mit YjjQ interagiert. Eine Analyse der Proteinstabilität von BglJ legte die Vermutung nahe, dass BglJ ein Substrat der Protease Lon und einer zweiten, unbekannten Protease ist. Die Vermutung, dass das Heterodimer BglJ-RcsB an eine Bindestelle in der Promotorregion des *bgl*-Operons bindet, wurde durch eine Analyse von *Linker*-Insertions-Mutanten der vermuteten Bindestelle unterstützt. Bindestudien mithilfe von DNA-Gelretardationsversuchen zeigten, dass die Bindung von RcsB an ein bekanntes Zielgen (*osmC*) durch die Anwesenheit von H-NS stimuliert wurde, während eine Bindung von BglJ-RcsB an die vermutete Zielsequenz am bgl-Operon in diesen Ansätzen nicht bestätigt werden konnte, da die Aufreinigung von BglJ-Protein misslang.

Desweiteren zeigten DNA-*Microarray*-Experimente, dass konstitutive Expression von BglJ zu einer signifikant höheren Transkription von Genen führte, die an zellulären Prozessen wie Säureresistenz, Stressantwort und Eisentransport beteiligt sind, sowie von Genen für Proteine der inneren und der äußeren Zellmembran. Diese Ergebnisse deuten auf eine globale Rolle von BglJ hin.

Zusammengefasst zeigen die Ergebnisse dieser Studie, dass BglJ und RcsB Heterodimere bilden können, welche die Repression des *bgl*-Operons durch H-NS aufheben können. Darüber hinaus wird deutlich, dass BglJ-RcsB eine wichtige Rolle als globaler Genregulator spielt.

I. Summary

The nucleoid-structuring protein H-NS is one of the most abundant DNA-binding protein in *Enterobacteriaceae*. H-NS plays a crucial role in the organization of the bacterial chromosome and in gene regulation. In *E. coli* H-NS regulates approximately 5% of genome by mostly acting as a transcriptional repressor. H-NS regulates gene expression in response to various environmental stimuli. H-NS act as a global transcriptional repressor by binding to specific sites followed by forming nucleoprotein complexes and DNA-H-NS-DNA bridges which prevent transcription initiation. H-NS mediated repression can be antagonized by specific transcription factors. This was shown for several H-NS repressed loci including the silent *bgl* operon in *E. coli*, which is a model system for studying H-NS mediated repression. Silencing of *bgl* is relieved by the LuxR-type transcription factor BglJ. This anti-silencing effect of BglJ is RcsB dependent. RcsB is a response regulator of a membrane stress induced Rcs signal transduction system which regulates various cellular processes in *Enterobacteriaceae*.

BglJ is a LuxR-type transcriptional regulator encoded in an operon with YjjQ. Previous two-hybrid analysis suggested that RcsB interacts with BglJ and also with YjjQ. In this present study the interaction of RcsB with BglJ and YjjQ was analyzed by Co-immunoprecipitation studies, which showed that RcsB interacts well with BglJ and weaker with YjjQ. Analysis of BglJ protein stability suggests that it is a target of Lon protease target and a second protease. Binding of BglJ-RcsB to a proposed putative RcsB/BglJ heterodimer binding site in the *bgl* regulatory region was supported by linker insertion mutant analysis in the putative binding site. Binding analyses by DNA shift assays indicate that binding of RcsB to a known target gene (*osmC*) at which act as homodimer is stimulated by H-NS, while binding analyses of BglJ-RcsB to its putative binding site in *bgl* could not yet be confirmed by DNA shift assays, as BglJ protein purification failed. In addition, DNA microarray experiments showed that constitutive expression of BglJ causes a significant increase in genes involved in acid resistance, stress response, iron transport, inner and outer membrane proteins suggesting a wide spread role of BglJ.

Taken together, the data support the model that RcsB forms a heterodimer with BglJ, that the RcsB-BglJ heterodimer antagonizes H-NS in *bgl*, and that it RcsB-BglJ plays an important role in global gene regulation.

II. Introduction

The genomic DNA in bacteria is associated with a class of DNA binding proteins referred as "Nucleoid-associated proteins" (NAP`s). The nucleoid-associated proteins play a crucial architectural role in the organization and compaction of the bacterial chromatin. *E. coli* consists of at least 12 distinct types of such proteins and each has its own characteristic expression pattern and DNA- binding preferences (Azam and Ishihama, 1999). The bacterial histone-like nucleoid structuring protein (H-NS) is one of the most abundant nucleoid-associated proteins found approximately 20000 copies per genome equivalent (Falconi et al., 1988). H-NS is highly pleiotropic and best characterized as a global repressor of transcription in Gram-negative bacteria (Dorman, 2009). Many transcriptional regulators have been shown to act as H-NS antagonists by disrupting the nucleoprotein complex. BglJ is one of such transcriptional regulator which counteracts H-NS mediated repression. Here in this present study, we showed the interaction of the BglJ with another transcription factor RcsB and the anti-repression of RcsB/BglJ heterodimer in H-NS repressed *bgl* operon.

1. H-NS, a nucleoid-associated protein

H-NS is a small basic protein of approximately of 15.6 kDa size, which is widespread in gram negative bacteria (Bertin et al., 2001; Tendeng and Bertin, 2003; Azam T.A et al., 1999; Falconi et al., 1988). It was first identified as a heat stable, low molecular weight DNA-binding factor in *E. coli* (Cukier-Kahn et al., 1972; Jacquet et al., 1971). Genomic and proteomic studies have shown that H-NS affects approximately 5% of the *E. coli* genes (Hommais et al., 2001). H-NS regulates gene expression in response to various environmental stimuli like temperature, osmolarity, acidic conditions and growth phase (White-Ziegler and Davis, 2009; Corbett et al., 2007; Amit et al., 2003; Dorman, 2009; Atlung and Ingmer, 1997). It was discovered recently that H-NS plays an important role in silencing horizontally acquired genes, including Pathogenicity Island encoding important virulence factors in *Escherichia coli* and *Salmonella enterica* (Grainger et al., 2006; Lucchini et al., 2006; Navarre et al., 2006; Navarre et al., 2007; Oshima et al., 2006; Wade et al., 2007). Increasing evidences also shows

that H-NS is involved in the regulation of bacterial biofilm formation (Gerstel and Romling, 2003; Vallet et al., 2004; Belik et al., 2008; Dalai et al., 2009).

2. H-NS mediated repression

H-NS is a pleiotropic regulator and mostly acts as a transcriptional repressor (White-Ziegler and Davis, 2009; Dame, 2005; Corbett et al., 2007) and (Dorman, 2007). The H-NS protein is 137 amino acids in length and has three domains (Fig. 1a). The amino terminal domain extending up to residue 65 is required for dimerization of H-NS; the carboxy terminal domain beginning at the residue 90 of the proteins has DNA binding activity. Both, the C and N terminal domains are connected by a highly flexible linker domain. This linker is required in formation of higher order oligomers of the protein. Dimerization, oligomerization and nucleic acid binding are crucial to the biological activity of H-NS (Dorman et al., 1999; Badaut et al., 2002; Esposito et al., 2002; Bloch et al., 2003).

H-NS preferentially binds to AT-rich and intrinsically curved sequences, which are commonly associated with promoters (Yamada et al., 1990; Yamada et al., 1991; Bracco et al., 1989; Jauregui et al., 2003). DNA foot printing and chip-on-chip studies have shown that H-NS binds to the AT-rich portions of the genomes of *Salmonella typhimurium* (Lucchini et al., 2006; Navarre et al., 2006) and *E. coli* (Grainger et al., 2006; Oshima et al., 2006). Only recently, a consensus sequence for H-NS was characterized (Bouffartigues et al., 2007; Lang et al., 2007; Dole et al., 2004; Nagarajavel et al., 2007). Repression of transcription by H-NS has been studied in detail at few promoters. These studies let to the following model; repression by H-NS is mediated by specific binding of the H-NS dimer to consensus sequence motifs which are also called nucleation sites. Then H-NS oligomerizes along the DNA and also forms DNA-H-NS-DNA bridges (Dorman and Kane, 2009). Formation of such complexes by H-NS results in DNA looping in which H-NA zips the two double strands that flank the promoter together. H-NS nucleoprotein complex formation may prevent binding of RNA polymerase or trap RNA polymerase at the promoter (Oshima et al., 2006; Noom et al., 2007; Dorman, 2007; Dame et al., 2006; Shin et al., 2005). The latter was shown in case of the P1 promoter of rrnB (rRNA encoding operon) and the *hdeAB* promoter (Shin et al., 2005).

For this promoter it was shown that H-NS binds to high affinity 'nucleation sites' and then interaction of H-NS dimers creates a nucleoprotein complex trapping RNA polymerase at the promoter (Fig 1b) (Rimsky et al., 2001; Rimsky, 2004; Dorman, 2004; Bouffartigues et al., 2007; Dame et al., 2006).

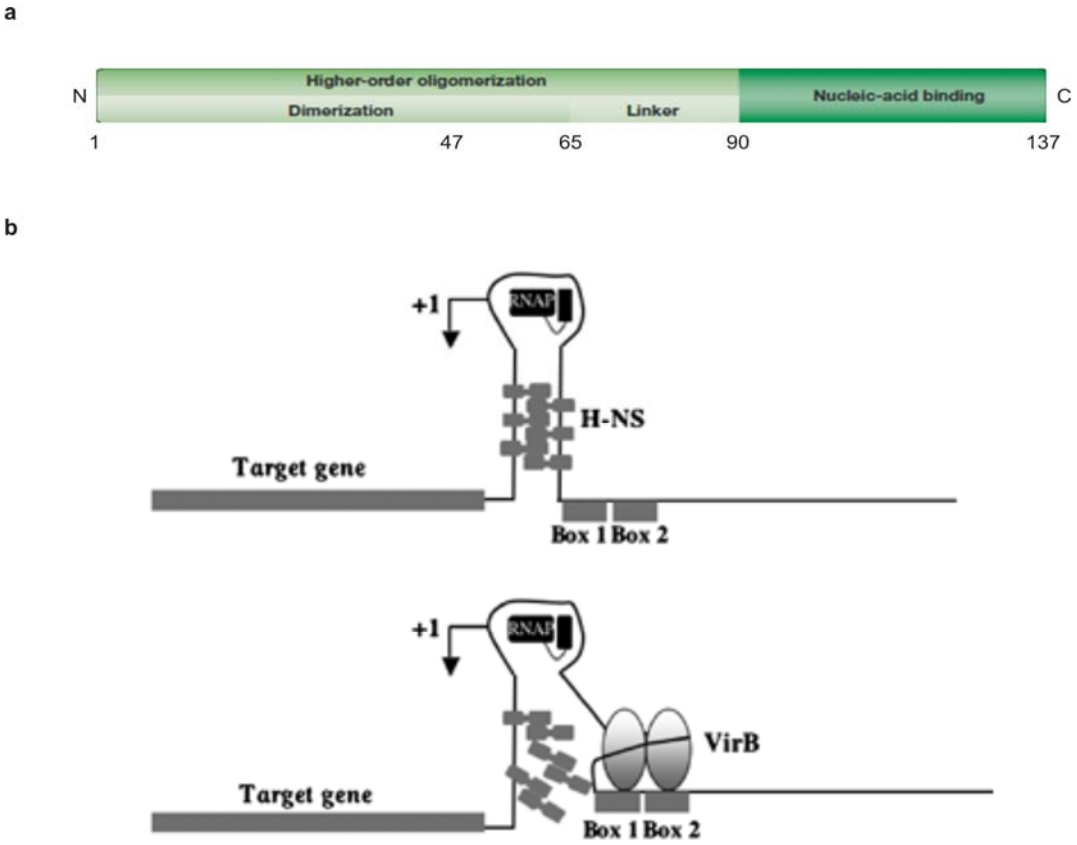


Fig 1. H-NS repression and antagonism of a DNA–H-NS–DNA bridge. **a)** The domain structure of H-NS is shown schematically. The numbers indicate the amino acid residues. N and C refer to N and C-terminal end of H-NS. The dimerization, linker and nucleic acid binding domains are indicated. **(b) Transcription repression and anti-silencing.** In the upper portion, the H-NS protein is shown cross linking two segments of DNA to form a repression loop at a bacterial promoter. RNA polymerase is trapped at the promoter and is unable to transcribe the gene that is the target of repression. In the lower part of the figure, the VirB regulatory protein binds to its recognition site, represented by the Box 1 and Box 2 motifs. DNA wrapping around the VirB dimer undermines the DNA–H-NS–DNA bridge and displaces H-NS. This liberates RNA polymerase from the repression complex and transcription of the target gene can commence (Dorman and Kane, 2009).

3. Anti-silencing of H-NS repression

H-NS mediated silencing can be relieved by the binding of specific transcription factors that disrupt or change the structure of the repressing nucleoprotein complex. Temperature-dependent alteration of the DNA structure and other changes in the physiological conditions that affect the DNA structure and DNA supercoiling at

specific loci can also relieve silencing by H-NS (Dorman, 2004; Navarre et al., 2007; Schroder and Wagner, 2002). Furthermore, repression by binding of H-NS within transcription units can be affected by the transcription activity (Nagarajavel et al., 2007). Many types of DNA-binding proteins can counter-act H-NS mediated silencing, as shown recently (Fang and Rimsky, 2008; Stoebel et al., 2008). The MarR family of transcriptional regulator SlyA counters H-NS mediated repression of the hemolysin gene *hlyE* in *E. coli* (Westermark et al., 2000; Lithgow et al., 2007). The LysR family regulator LeuO counteracts H-NS-mediated repression of specific loci in *Salmonella enterica* and in *E. coli* (Chen et al., 2005; Fernandez-Mora et al., 2004; Madhusudan et al., 2005; Stratmann et al., 2008). The regulatory protein TraJ counter-acts H-NS repression of the *tra* genes in the F-plasmid of *E. coli* (Will and Frost, 2006),. Ler, a homolog of H-NS encoded by LEE (Locus of Enterocyte Effacement) pathogenicity island in EHEC (enterohemorrhagic *E. coli) EPEC* (Enteropathogenic *E.coli*) antagonizes the H-NS repression (Torres et al., 2008; Williamson and Free, 2005). ToxT, an AraC like transcription factor antagonizes H-NS mediated silencing at the *ctx* and *tcpA* promoters in *Vibrio cholarae* (Nye et al., 2000; Yu and DiRita, 2002). Other nucleoid-associated protein can also antagonize H-NS repression, the heat unstable nucleoid protein (HU) can compete with H-NS for the same binding sites in the promoter region of the test DNA (pSFV1) (van et al., 2004). Similarly, Fis protein (factor for inversion stimulation) has been reported to antagonize H-NS repression at rRNA promoter (Schneider et al., 2003). Our group is more focused on studying the mechanism of H-NS mediated repression using *bgl* operon as a model.

4. *bgl* operon

The *bgl* operon encodes gene products necessary for the uptake and fermentation of aryl D-glucosides like arbutin and salicin. The *bgl* operon consists of six genes namely *bglG*, *bglF*, *bglB*, *bglH*, *bglI* and *bglK* (Fig 2). Two Rho-independent transcriptional terminators, *t1* and *t2*, flank the first gene of the operon, which encodes an antiterminator, BglG (Prasad and Schaefler, 1974; Schaefler and Maas, 1967; Mahadevan and Wright, 1987; Schnetz et al., 1987; Schnetz and Rak, 1988).

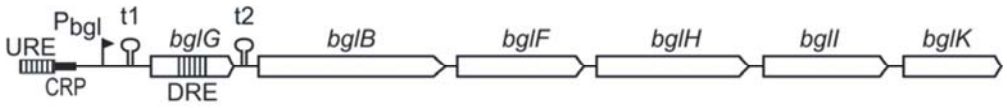


Figure 2. The *E. coli bgl* operon. Scheme showing the *bgl* operon with the promoter (P_{bgl}), the CRP binding site (CRP), the Rho independent terminators (t1 and t2) and the structural genes *bglG,B,F,H,I* and *K* (bglG, transcriptional antiterminator, BglB, phospho β-glucosidase, bglF, EII permease, bglH, outer membrane porin, bglI xylanase, bglK, isomerase). The H-NS binding sites in URE (upstream regulatory element) and DRE (downstream reualtory element) are indicated with vertical hatched bars.

The *bgl* operon is present in three of the four phylogenetic groups of *E. coli* including commensals and pathogens, and in all strains examined the *bgl* operon is silenced by H-NS (Sankar et al., 2009). Interestingly, in uropathogenic and septicemic isolates silencing of *bgl* is less strict. In *E. coli* K12 wild type, the *bgl* operon is transcriptionally repressed (~100-fold) by the histone-like nucleoid associated protein. So far, no laboratory conditions has been established for the activation of *bgl* operon (Schnetz, 1995; Higgins et al., 1988; Dole et al., 2004; Nagarajavel et al., 2007). H-NS binds to the upstream regulatory element (URE) located immediately upstream of the cAMP receptor protein (CRP)-dependent promoter and within a downstream regulatory element (DRE) +600 to +700 bp downstream of the transcription start site (Schnetz, 1995), (Dole et al., 2004) and mediates the silencing of the operon. H-NS mediated repression is relieved by the transcriptional regulators LeuO and BglJ, both of which presumably bind to the URE and counteract H-NS mediated repression (Ueguchi et al., 1998; Madhusudan et al., 2005). Recently, it was discovered in the lab that anti-silencing of the *bgl* operon by BglJ requires RcsB (Paukner, 2007). RcsB is the response regulator of the Rcs signaling system (see below). Two-hybrid analysis suggest that BglJ and RcsB form heterodimers (unpublished data of the lab).

In addition to BglJ and LeuO, various spontaneous mutations, which map close to the CRP-dependent promoter, including the deletion of an AT-rich regulatory region upstream of the promoter, integration of insertion elements, and point mutations that improve the CRP-binding site relieve silencing, presumably by disrupting the repressing nucleoprotein complex formed by H-NS (Schnetz and Rak, 1992; Mukerji and Mahadevan, 1997). Once activated, transcription from the *bgl* promoter initiates constitutively. However, the operon is still regulated substrate specifically. In the absence of inducer, the majority of transcripts terminate prematurely at one of the two rho-independent terminators within the operon, whereas, in the presence of substrate,

transcription proceeds through both terminators to the ends of the operon (Schnetz and Rak, 1988; Amster-Choder, 2005).

5. Rcs two-component system

In prokaryotes, two-component systems (TCS) are widespread signal transduction devices that enable the bacteria to elicit an adaptive response to environmental stimuli, mainly through changes in gene expression. The Rcs phosphorelay is a complex, two-component system originally identified in *E. coli* as a regulator of the expression of *cps* operon, encoding the proteins required for the production of capsular polysaccharide colonic acid (Gottesman et al., 1985). The Rcs system is a membrane stress response signalling device exclusively found in enterobacteriaceae family (Conter et al., 2002; Erickson and Detweiler, 2006; Kaldalu et al., 2004; Sailer et al., 2003; Ebel et al., 1997; Ize et al., 2004; Parker et al., 1992; Shiba et al., 2004; Laubacher and Ades, 2008). Unlike other TCS, the Rcs system is composed of three proteins (Fig. 1). RcsC is an inner membrane located hybrid sensor kinase with a conserved histidine kinase domain (H) and a receiver domain (D). The second protein, RcsD, consists of a histidine phosphotransfer domain (Hpt) but lacking a histidine domain (Fig 3). RcsB, the response regulator consists of a conserved N-terminal receiver domain and a C-terminal DNA binding helix-turn helix domain.

Upon sensing of an environmental signal, the conserved histidine in the kinase domain (H) of RcsC is phosphorylated by an autophosphorylation event and the phosphoryl group is transferred to the RcsC receiver domain (D). The phosphoryl group is then transferred to the conserved histidine in the Hpt domain of RcsD and finally to the receiver domain of RcsB. The phosphorylated RcsB regulates the target genes as a homodimer and also can form heterodimer with an auxiliary protein, RcsA, and regulate target genes. Transcriptome analyses suggest that up to 2.5% of the *E. coli* genome might be regulated by the Rcs system (Ferrieres and Clarke, 2003); Hagiwara et al., 2003). The Rcs system regulates the transcription of wide range of genes, including those encode the exopolysacharide synthesis operon (*cps* and *yjbEFGH* (Gottesman et al., 1985; Ferrieres et al., 2007), the cell division genes (*ftsA* and *ftsZ,* (Carballes et al., 1999) osmoregulated genes (*osmB,* (Boulanger et al., 2005); *osmC,* (Davalos-Garcia et al., 2001), flagellar biosynthesis genes (*flhDC*, (Francez-Charlot et al., 2003), stress response sigma factor σ^S (*rprA,* (Majdalani et

al., 2002), curli synthesis operon (c*sgDEFG,* (Vianney et al., 2005), and genes involved in biofilm formation (*bdm,* (Francez-Charlot et al., 2005).

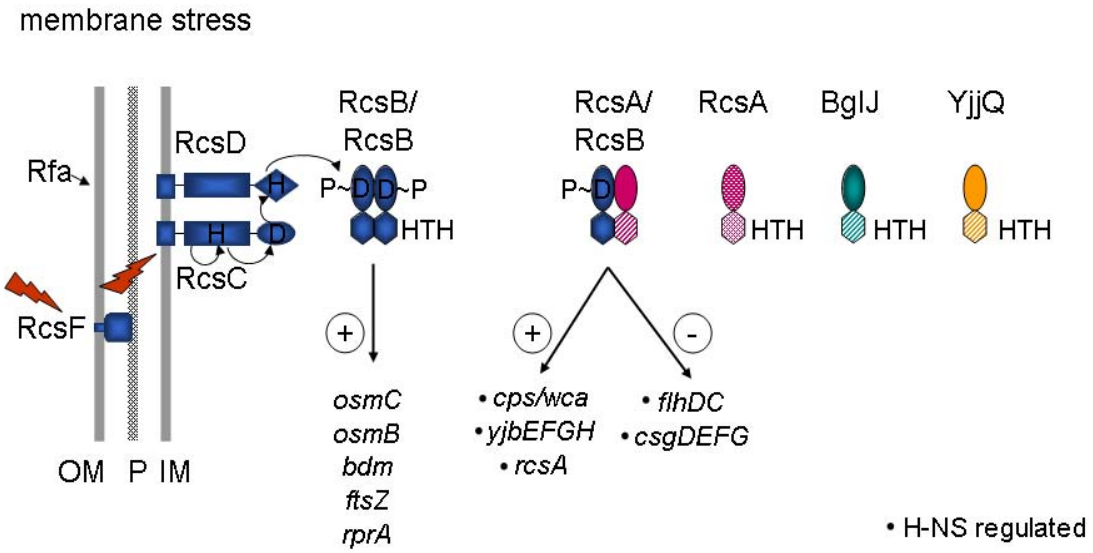


Fig 3. Model of signal transduction pathway for Rcs signal transduction system. The Rcs proteins RcsB,C,D with RcsF is shown. The kinase domain with conserved histidine (H) and receiver domain (D) with conserved aspartate and the phosphorylation process is shown. The jagged arrows indicate signals coming from outside the cell. The lipid biosynthesis protein (Rfa) is shown. The RcsB homodimer target genes and RcsB/RcsA heterominer target genes are shown. The + and – signs indicate positive and negative regulations respectively. P stands for phosphorylated form of the particular protein, in this case RcsB. The RcsB binding partners RcsA, BglJ and YjjQ are shown. The helix-turn helix motif is also mentioned. The black dots represent the H-NS regulated genes.

6. *yjjQ-bglJ* operon

The *yjjP-yjjQ-bglJ* operon is present in the enterobacterial species *E. coli* (including the *Shigella* spp.) and *S. enterica*. The *yjjQ* and *bglJ* genes are arranged in tandem with overlapping open reading frames. They belong to LuxR-type family of transcription factors with a typical DNA-binding helix-turn-helix *(*HTH*)* motif in the C-terminal domain (Fig 4). The This operon is repressed by the global regulator H-NS and the repression is counteracted by the LsyR-type transcriptional regulator LeuO (Chen et al., 2005; Madhusudan et al., 2005; Stratmann et al., 2008). The disruption if *yjjQ* by a transposon insertion resulted in attenuation of virulence in avian pathogenic *E. coli* (APEC) (Li et al., 2005).

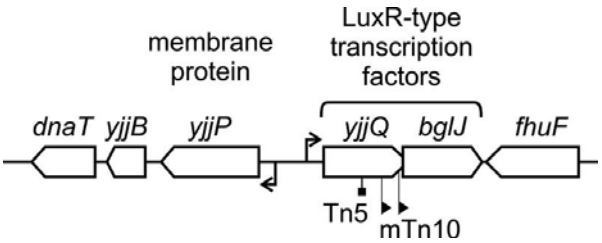


Fig 4. Organization of the *yjjP-yjjQ-bglJ* locus. The operon mapping at 99 min of the *E. coli* K-12 genome in between *yjjB* (encoding a conserved inner membrane protein) and *fhuF* (encoding a ferric iron reductase protein). The *yjjQ* and *bglJ* genes encode LuxR-type transcription factors. A *yjjQ*::Tn*5* insertion mutation attenuates the virulence of APEC (Li et al., 2005), while mini-Tn*10* insertions upstream of *bglJ*, causing the constitutive expression of *bglJ*, relieve the silencing of the *bgl* operon by H-NS in *E. coli* K-12 (Giel et al., 1996; Madhusudan et al., 2005). The *yjjP* gene encodes a membrane protein of unknown function (Daley et al., 2005). (Figure from Stratmann et al., 2008).

The *yjjQ* mutants were negatively selected in a genome-wide screen for Salmonella genes required for long-term systemic infection of the mouse (Lawley et al., 2006). A constitutively expressing *bglJ* (due to mini-Tn10 insertion) in *E. coli* K-12 have shown to de-repress H-NS regulated *bgl* operon (Giel et al., 1996). BglJ forms heterodimer with another transcriptional regulator RcsB and de-represses H-NS regulated *bgl* operon in *E. coli* (This study and unpublished data). The RcsB/BglJ heterodimer also counteract the repression of H-NS and activate the leuO (Schnetz K, unpublished data).

7. Aim of the thesis

The aim of the study is to understand the mechanism of anti-repression of H-NS regulated *bgl* operon silencing by the transcriptional factor BglJ and its dependence on RcsB. Firstly, heterodimerization of BglJ and RcsB was analyzed by Co-immunoprecipitation. These experiments included analyses of YjjQ, which is encoded with BglJ in one operon. Here we showed that the newly identified Lux-R type transcriptional regulators, BglJ and YjjQ interact with RcsB, the response regulator of Rcs signaling system. Secondly, the binding of RcsB-BglJ to the *bgl* URE was analyzed and a putative binding site for RcsB-BglJ was mapped. I also showed the binding of RcsB-BglJ may require H-NS as a necessary factor.

III. Results

1. Interaction of RcsB with BglJ and YjjQ

Two-hybrid analyses had demonstrated that RcsB and BglJ interact. The first part of the present study focused on showing the interaction of RcsB with BglJ and YjjQ, respectively through biochemical analysis. To confirm the interaction I performed Co-immunoprecipitation assays. In a first step the expression of C-terminally epitope tagged BglJ and RcsB variants were tested. Then BglJ-Flag and RcsB-HA were co-expressed and the interaction was analyzed by immunoprecipitation with an HA specific antibody. Similarly, the interaction of RcsB and YjjQ was analyzed.

1.1 Expression of epitope tagged proteins

For the expression of epitope tagged BglJ protein, the *bglJ* gene was cloned into a set of plasmids which carry the inducible *Ptac* promoter followed by a multiple cloning site and a sequence encoding either a Myc-tag, a FLAG-tag, or a HA-tag (Fig 5). For efficient translation the plasmids carry the extended Shine-Dalgarno sequence SD_{gene10} derived from phage T7 gene 10 to which the *bglJ* and *yjjQ* genes were fused. Upstream of the *tac* promoter maps the $lacI^q$ gene. Low and high copy variants of these vectors were used for cloning of the *bglJ* and *yjjQ* gene with a fusion of a tag sequences at the 3' end (Fig 5).

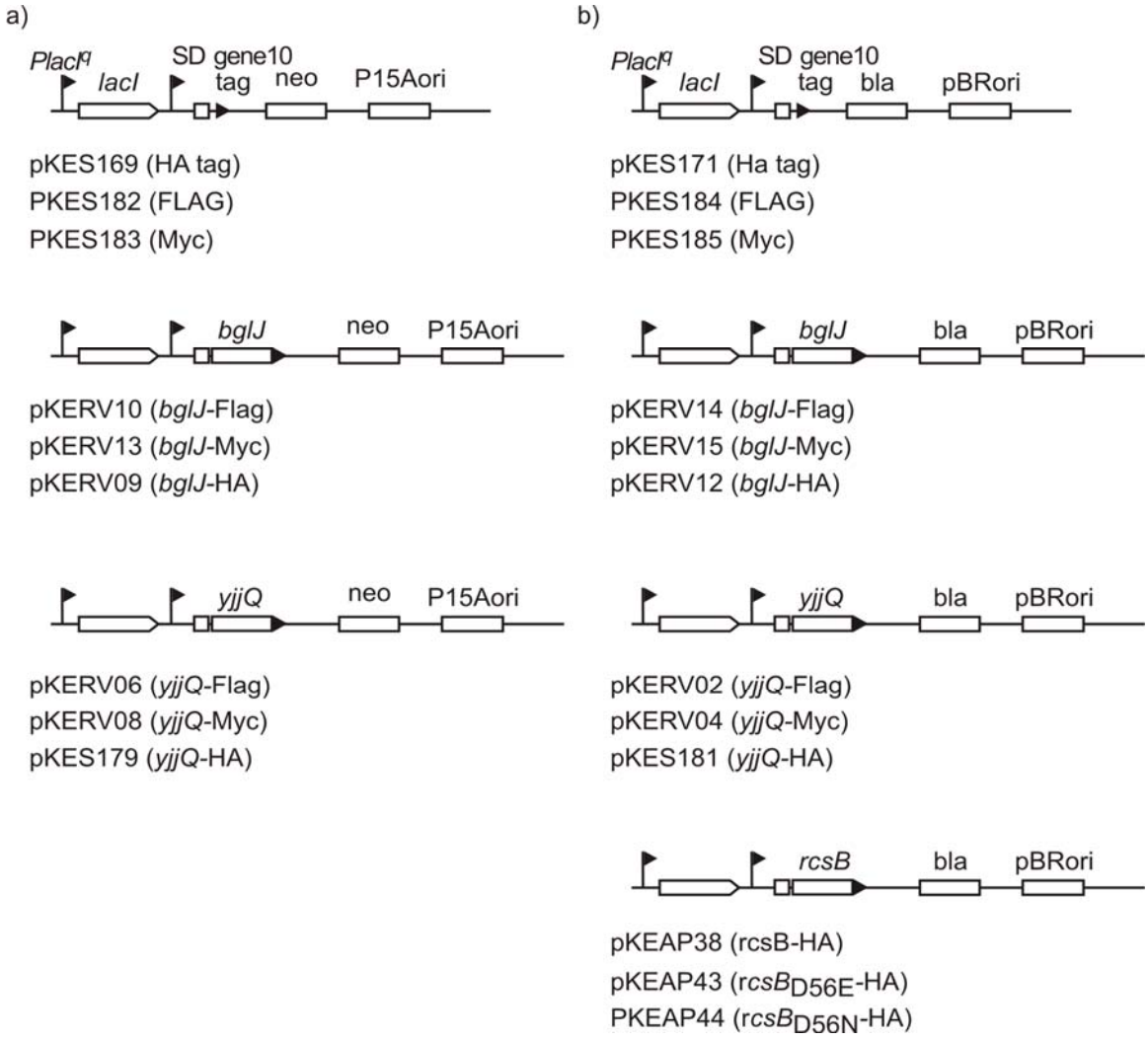


Fig 5. Plasmids for expression of C-terminal Flag/Myc/HA tagged BglJ and YjjQ proteins and HA-tagged RcsB protein. Schematic representation of plasmids for expression of C-terminally tagged BglJ, YjjQ, and RcsB variants (a,b). The plasmids carry a lacI^q gene followed by the IPTG inducible *tac* promoter, a strong phage T7 gene Shine-Dalgarno sequence and a multiple cloning site for cloning of *bglJ* and *yjjQ* fusions with a tag (Flag/Myc or HA) at the 3`end. The low copy plasmids (a) carry a p15A origin and a kanamycin resistance gene. The high copy plasmids (b) carry a pMB1 origin and ampicillin resistance marker. (a) The low copy plasmids pKES169, pKES183, and pKES182 were used for cloning and pKES169 was used as empty control vector in expression studies. Similarly, high copy plasmids pKES171, pKES184, and pKES185 were used for cloning of the high copy vectors for expression of tagged *bglJ* and *yjjQ*. Plasmid pKES171 was used as control plasmid in expression studies. For expression of RcsB and its mutants high copy number similar plasmids were used which carry *rcsB* with its native Shine-Dalgarno fused to a HA-epitope encoding sequence at their 3' end (Paukner A, 2007). The plasmid numbers and encoded genes are schematically represented and the cloning is documented in laboratory database.

The expression and solubility of the BglJ-HA, BglJ-FLAG, and BglJ-Myc from these plasmids was tested by Western blots (Fig 6). The expression of the BglJ-FLAG and BglJ-Myc tagged fusion proteins was marginally higher when directed by the high copy plasmid variant than low copy variants (Fig 6a and c). However, in the soluble

fraction of a cell free protein extract BglJ-FLAG and BglJ-Myc tagged protein levels showed no significant difference between the low and high copy plasmid variants than the low copy variants (Fig 6b and d). The expression of the HA tagged BglJ fusion protein was weaker than that of the FLAG or Myc tagged BglJ protein (Fig 6e and f). Similarly, The expression of YjjQ-FLAG, Myc and HA tagged fusion proteins was higher in the high copy plasmid variants (Fig 6a, b and c). The soluble fraction of a cell free protein extract YjjQ-FLAG and YjjQ-Myc tagged protein levels were high and showed no significant difference between the low and high copy plasmid variants. The YjjQ-HA tagged protein levels were weaker in low and high copy variants than FLAG and Myc tagged variants (Fig. 6b, d and f).

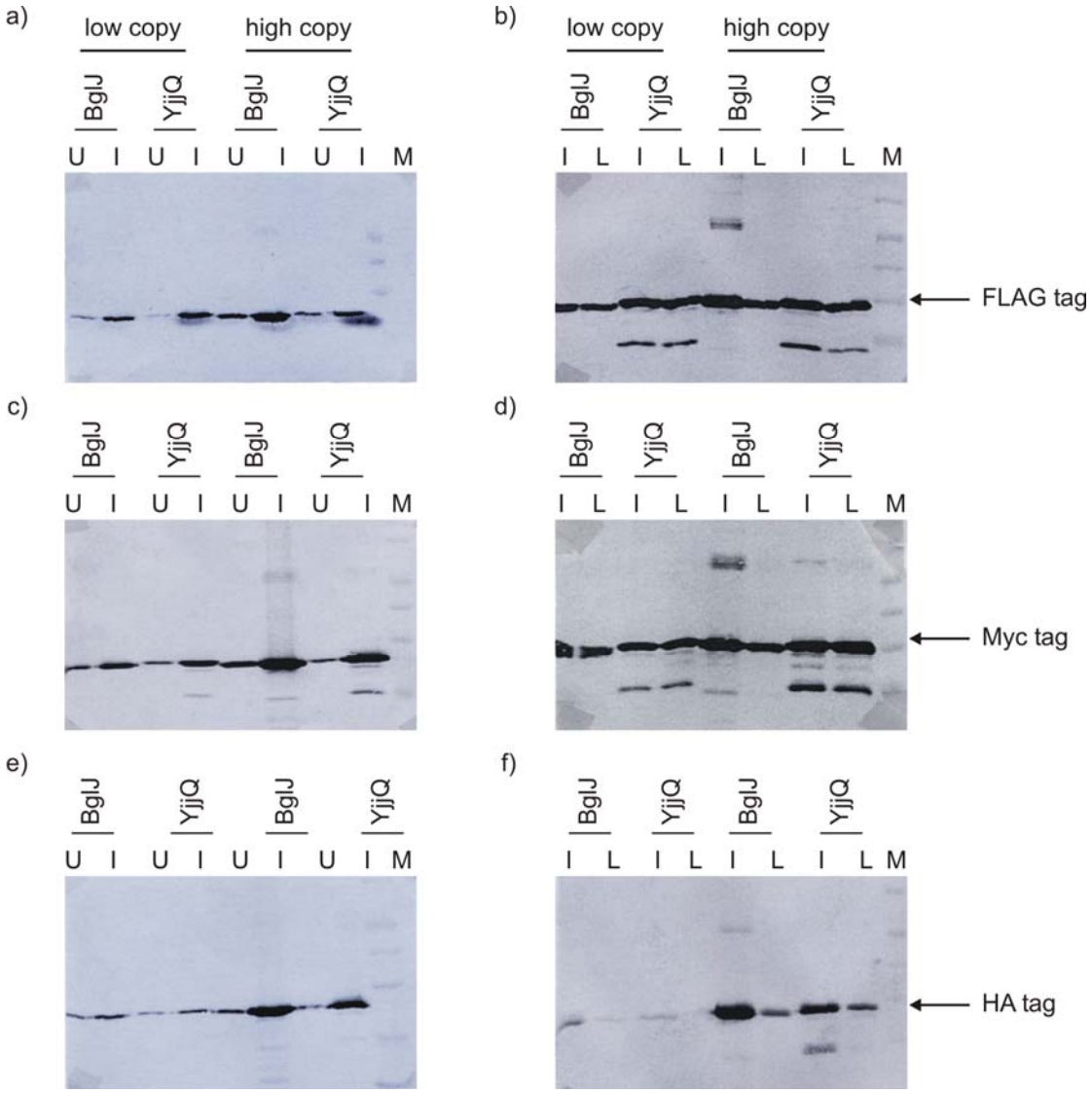


Fig 6. Expression of FLAG/Myc/HA tagged BglJ/YjjQ and HA-tagged RcsB proteins. For expression analyses of plasmids encoding the tagged variants of BglJ, YjjQ, and HA were transformed into *E. coli* strain S3377 carrying deletions of the *yjjQ-bglJ* and *rcsB* genes. Transformants were grown at 37°C in LB with antibiotics to OD600=0.3 and protein expression was induced for 2 hours with 1mM IPTG. For analysis of induction samples from uninduced (U) and induced (I) cultures were resolved on 12% SDS-PAGE and analyzed by Western blotting. Rat-anti HA and anti-rat alexaflour®680 antibodies were used for HA tagged proteins. Mouse-anti FLAG and mouse-anti-Myc antibodies with anti-mouse alexaflour®680 antibodies were used for FLAG and Myc tag proteins. For analysis of the solubility of the proteins a cell free protein lysate (L) was loaded next to the samples of induced cultures (I). Each lane was loaded with $0.05OD_{600}$ cells. The blots were visualized on an Odyssey infrared imaging scanner. Low copy plasmids used were BglJ-FLAG (pKERV10), BglJ-Myc (pKERV13), BglJ-HA (pKERV9), YjjQ-FLAG (pKERV6), YjjQ-Myc (pKERV8), and YjjQ-HA (pKES179.), High copy plasmids used were BglJ-FLAG (pKERV14), BglJ-Myc (pKERV15), BglJ-HA (pKERV12), YjjQ-FLAG (pKERV2), YjjQ-Myc (pKERV4), YjjQ-HA (pKES181) RcsB-HA (pKEAP38), $RcsB_{D56N}$-HA (pKEAP44), and $RcsB_{D56E}$-HA (pKEAP43) (See Materials and methods V.4). The ~27 kDa BglJ and 28 kDa YjjQ proteins with the respective epitope tags are indicated by an arrow.

For expression of HA tagged RcsB and its mutants $RcsB_{D56E}$ and $RcsB_{D56N}$ high copy plasmids of similar structure were used, which carry *rcsB* and its mutants with their native Shine-Dalgarno sequence (Fig 5). Upon induction the expression of RcsB and its mutants $RcsB_{D56E}$ and $RcsB_{D56N}$ was similar (Fig 6g). Western analysis of the soluble fraction of cell free extract showed the wild type RcsB levels were higher than the levels of its mutants (Fig 6 h).

Based on the observed protein yields in the lysates, I decided to use low copy plasmids expressing BglJ-FLAG (pKERV10) and YjjQ-FLAG (pKERV06). For co-expression of RcsB, I used the high copy plasmid pKEAP38, encoding C-terminal HA tagged RcsB, and plasmids pKEAP43 and pKEAP44 encoding $rcsB_{D56E}$ -HA and $rcsB_{D56N}$ -HA, respectively.

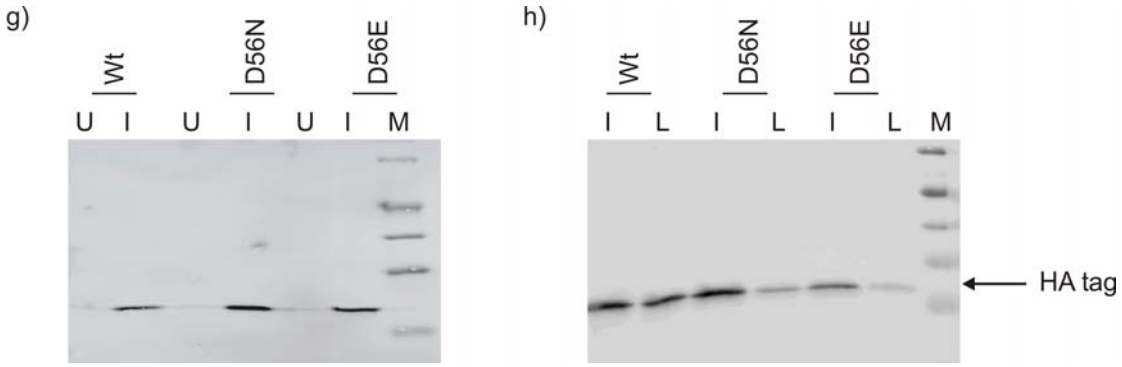
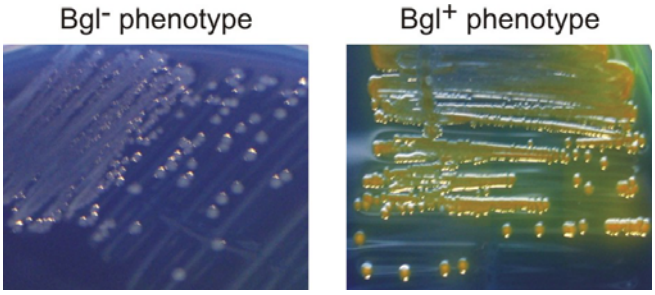


Fig 6. Expression of FLAG/Myc/HA tagged BglJ/YjjQ and HA-tagged RcsB proteins continued. The ~25 kDa HA-tagged RcsB and mutant proteins are indicated by an arrow.

1.2 Phenotype analysis

The functionality of the C-terminal FLAG/HA/Myc tagged BglJ and HA-tagged RcsB and their mutant was checked with a phenotype assay. In *E. coli* K12 utilization of β-glucosides like arbutin and salicin requires the expression of the *bgl* operon. Wild type *E. coli* are phenotypically Bgl^- due to H-NS silencing. An *E. coli* strain S2828 which carries a mini transposon insertion within the *yjjQ-bglJ* operon causing constitutive expression of BglJ (S2822) in addition to a *rcsB* gene deletion (Paukner, 2007) is transformed with RcsB and the mutants and plated on a BTB salicin plates. Similarly, the C-terminal FLAG/HA/Myc tagged BglJ was tested in *E. coli* S524, which is phenotypically Bgl^-. The plates were incubated at 37ºC, for β-glucoside

utilization. The strain carrying plasmid control remained Bgl^- and the strain carrying RcsB and mutants showed Bgl^+ phenotype after 1 day of incubation (Fig 7).



strain	plasmids	genotype/description	phenotype
S2822	pKEAP22	yjjQ/bglJ-Y6::mTn10cm	+
S2828	pKEAP22	S2822 rcsB::mTn10tet	-
S2828	pKEAP22	Vector control	-
S2828	pKEAP38	RcsB-HA	+
S2828	pKEAP43	$RcsB_{D56E}$-HA	+
S2828	pKEAP44	$RcsB_{D56N}$-HA	+
S524	pKES169	Vector control	-
S524	pKERV09	bglJ-HA	+
S524	pKERV10	bglJ-FLAG	+
S524	pKERV13	bglJ-MYC	+

Fig 7. Bgl phenotype assay with BTB salicin plates for β-glucoside utilization. *E. coli* strain S2828 (S524 rcsB::mTn10tet yjjQ/bglJ-Y6:: mTn10cm) was complemented with RcsB-HA (pKEAP38), $RcsB_{D56E}$-HA (pKEAP43), $RcsB_{D56N}$-HA (pKEAP44) and a vector control (pKEAP22). The plasmids coding for C-terminal HA/FLAG/Myc tagged BglJ (pKERV09, pKERV10, pKERV13) and vector control (pKES169) were transformed into *E. coli* S524 strain and plated on a BTB salicin plate. The plates were incubated at 37ºC for one day. The Bgl^- phenotype was shown in blue background and the Bgl^+ phenotype was shown in yellow background.

1.3 Interaction of RcsB with BglJ

The interaction of RcsB with BglJ was carried out by co-immunoprecipitation. An *E. coli* strain S3377 (ΔrcsB::SpecR Δ(yjjP-bglJ)::KD3cmR) carrying deletions of the *yjjQ-bglJ* and *rcsB* genes was transformed with the two plasmids encoding RcsB-HA (pKEAP38) and BglJ-FLAG (pKERV10). As controls, single transformants with only one of the plasmids and the non-transformed strain were used. Cells were grown to early exponential phase (OD_{600}=0.3) and then protein expression was induced with 1mM IPTG for 2 hours. The cell lysate was prepared by sonication and 200µg of the total protein was used for the immunoprecipitation assay.

The interaction between RcsB-HA and BglJ-FLAG was determined by co-immunoprecipitation of cell lysates with rabbit anti-HA IgG antibody in conjunction with protein-A sepharose (see Materials and Methods.14). The lysates and the

precipitates were separated on 12% SDS-gels and analyzed by a Western blot. The Western blot was developed using rat-anti HA and anti-rat alexaflour@680 antibodies for HA tagged proteins. Mouse-anti FLAG and anti-mouse alexaflour@800 antibodies were used for FLAG tagged proteins. The lysate (expression control) showed that all the proteins were expressed (Fig 8a, lane 1-4). The co-immunoprecipitate with rabbit anti-HA IgG showed the presence of BglJ-FLAG with RcsB-HA when both proteins were co-expressed (Fig 8a, lane 8). BglJ-FLAG was not detected in the precipitate, when it was expressed alone (Fig 8a, lane 7). The controls were as expected, no bands were visible in the lysate and precipitate of the non-transformed bacteria (Fig 8a, lane 1 and 5), and RcsB-HA was expressed and immunoprecipitated when it was expressed alone (Fig 8a, lanes 2 and 6). These data show that RcsB-HA protein and BglJ-FLAG interact specifically. The protein ratio of RcsB-HA and BglJ-FLAG in the lysate and the immunoprecipitate remained the same, indicating that the interaction is efficient.

Similarly, I performed co-immunoprecipitation of BglJ-FLAG with a mutant $RcsB_{D56E}$-HA protein (Fig 5). The multi-component Rcs phosphorelay signaling pathway absolutely requires its response regulator RcsB to regulate all its target genes. The mutant variant $RcsB_{D56E}$, with the conserved aspartate residue at position 56 replaced by a glutamate residue, was isolated during a mutation analysis (Gupte et al., 1997). The mutated aspartate residue is conserved within the receiver domain of the family of bacterial response regulator proteins (Parkinson and Kofoid, 1992) and has been shown to be the site of phosphorylation in several response regulators (Keener and Kustu, 1988; Klose et al., 1993). The $RcsB_{D56E}$ variant mimics the phosphorylated form of the protein and activates the transcription of capsular polysaccharide (*cps*) genes constitutively (Stout, 1994). This mutant variant showed similar activity like the wild type when tested for heterodimer formation with BglJ using a bacterial two-hybrid system (unpublished lab data). So, we were interested to test the relevance of the variant in the *in vitro* analysis. In the co-immunoprecipitation assay with rabbit anti-HA IgG antibody BglJ-FLAG protein was co-precipitated with $RcsB_{D56E}$-HA demonstrating interaction of $RcsB_{D56E}$ and BglJ proteins. Comparison of the protein ratio of $RcsB_{D56E}$ and BglJ in the lysate and precipitate indicates that the co-immunoprecipitation of BglJ with $RcsB_{D56E}$ was less efficient than with wild-type RcsB. However, this experiment was performed only once.

In addition, I performed a co-immunoprecipitation assay of BglJ-FLAG with $RcsB_{D56N}$ (Fig 5). $RcsB_{D56N}$ is a mutant in which the conserved aspartate residue at position 56 was replaced by an aspargine residue. This mutation mimics the non-phosphorylated form of RcsB protein which might be inactive as transcriptional regulator (Gupte et al., 1997). No co-precipitation of BglJ-FLAG protein with $RcsB_{D56N}$-HA was observed when precipitated with rabbit anti-HA IgG antibody. This indicates a lower stability of the BglJ-FLAG and $RcsB_{D56N}$ interaction. However, this experiment was performed only once and expression of the protein was poor. Also, the results contradict results of two-hybrid analyses performed previously in the lab (unpublished lab data).

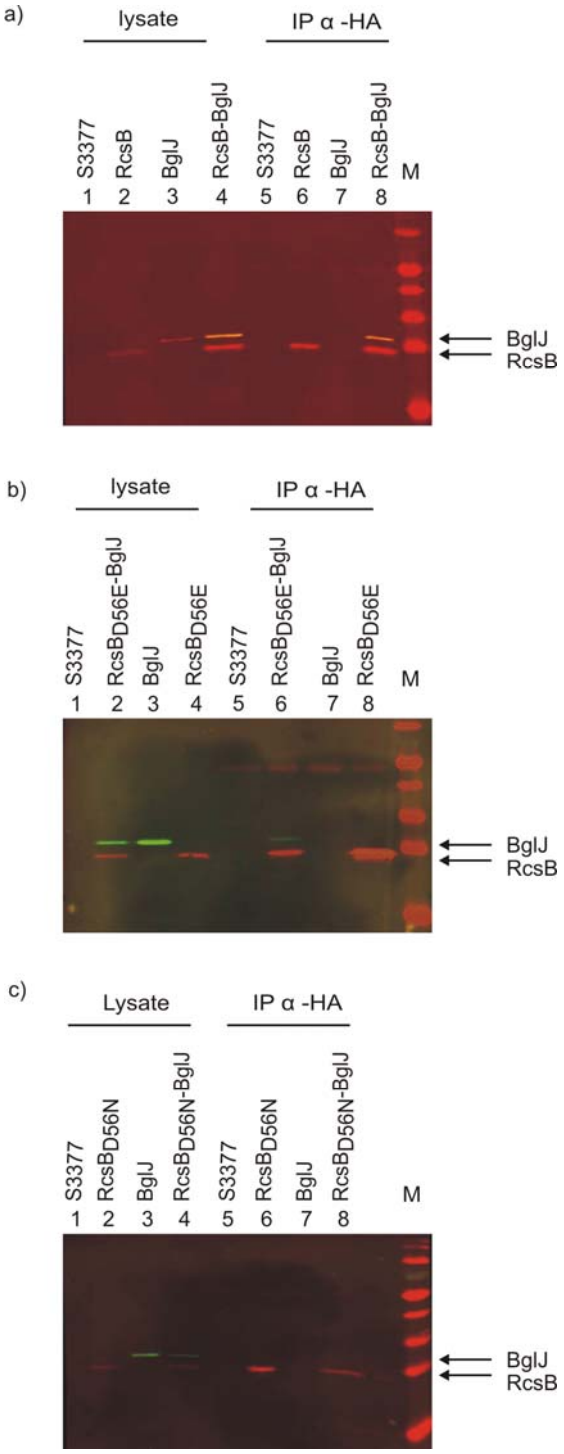


Fig 8. Interaction of RcsB, $RcsB_{D56E}$, and $RcsB_{D56N}$ with BglJ analyzed by co-immunoprecipitation.
Transformants of *E .coli* strain S3377 (Δ*rcsB*, Δ(*yjjP-yjjQ-bglJ*) with plasmids expressing C-terminal HA tagged RcsB and BglJ-FLAG. Cell lysates were prepared after induction of protein expression with 1mM IPTG for 2hrs at 37°C. 200µg of the total protein from the lysates expressing neither of the proteins (S3377), RcsB-HA or BglJ-FLAG alone, are both proteins were precipitated with rabbit-anti-HA IgG antibody in conjunction with protein-A sepharose. The precipitates and the lysate were separated on a 12% SDS gel and blotted for Western analysis. The Western blot was analyzed using rat-anti HA and anti-rat alexaflour@680 antibodies (red color) for HA tagged proteins. Mouse-anti-FLAG and anti-mouse alexaflour@800 antibodies (green color) were used for FLAG tagged proteins. The blot was developed using an Odyssey scanner at 700 and 800nm channels. (a) immunoprecipitation of RcsB-HA (pKEAP38, high copy) with BglJ-FLAG (pKERV10, low copy), (b) $RcsB_{D56E}$-HA (pKEAP43, high copy) with BglJ-FLAG, (c) $RcsB_{D56N}$-HA (pKEAP44, high copy) with BglJ-FLAG (see Materials and methods. 6). RcsB and BglJ protein are indicated by an arrow.

1.4 RcsB interaction with YjjQ

As for interaction of RcsB with YjjQ by co-immunoprecipitation, the same protocol was used. *E. coli* strain S3377 (Δ*yjjP-yjjQ-bglJ*), Δ*rcsB*) was transformed with two plasmids encoding RcsB-HA (pKEAP38) and YjjQ-FLAG (pKERV6). As controls,

single transformants with only one of the plasmids and the non-transformed strain were used. The cell lysates were immunoprecipitated with Rabbit anti-HA IgG antibody in conjunction with protein-A sepharose and the lysates and precipitates were analyzed by western blotting using tag specific antibodies (see Materials and Methods14). The lysate (expression control) showed that all the proteins were expressed (Fig 9a, lanes 1-4). The co-immunoprecipitate with Rabbit-anti-HA IgG showed the presence of YjjQ-FLAG with RcsB-HA when both the proteins were co-expressed (Fig 9a, lane 8). As expected, YjjQ-FLAG was not detectable in the precipitate, when it was expressed alone (Fig 9a, lane 7). Similarly, in the lysate and precipitates of the non-transformed strain no proteins were visible (Fig 9a.5). RcsB-HA was expressed and immunoprecipitated when it was expressed alone (Fig 9a, lanes 2 and 6). These data show that RcsB-HA protein and YjjQ-FLAG interact specifically. A 3.2 fold higher precipitation of BglJ protein than YjjQ indicates that BglJ interacts stronger than YjjQ with RcsB (The BglJ and YjjQ values obtained from band intensities which were normalized to RcsB). This result also supports the bacterial-two hybrid data which suggested that the interaction of RcsB with BglJ is stronger than with YjjQ (unpublished data of the group).

In addition, I performed co-immunoprecipitation of YjjQ-FLAG with the mutant proteins $RcsB_{D56E}$-HA and $RcsB_{D56N}$-HA. YjjQ-FLAG co-precipitated with the D56E mutant and the protein ratio was similar to the wild type indicating that the mutation has no significant effect in interaction with YjjQ protein. In contrast, no precipitation of YjjQ-FLAG with $RcsB_{D56N}$-HA with rabbit anti-HA IgG antibody was observed. This indicates that the affinity of YjjQ-FLAG to the mutant $RcsB_{D56N}$ which mimics the non-phosphorylated (i.e inactive) form of RcsB is lower. The co-immunoprecipitation experiments of YjjQ-FLAG with wild-type and mutant RcsB-HA protein was performed only once, but the results are in agreement with two-hybrid analyses performed earlier. In the two-hybrid analysis interaction of YjjQ with wild-type RcsB or with $RcsB_{D56E}$ was significantly more efficient than with $RcsB_{D56N}$ (unpublished lab data).

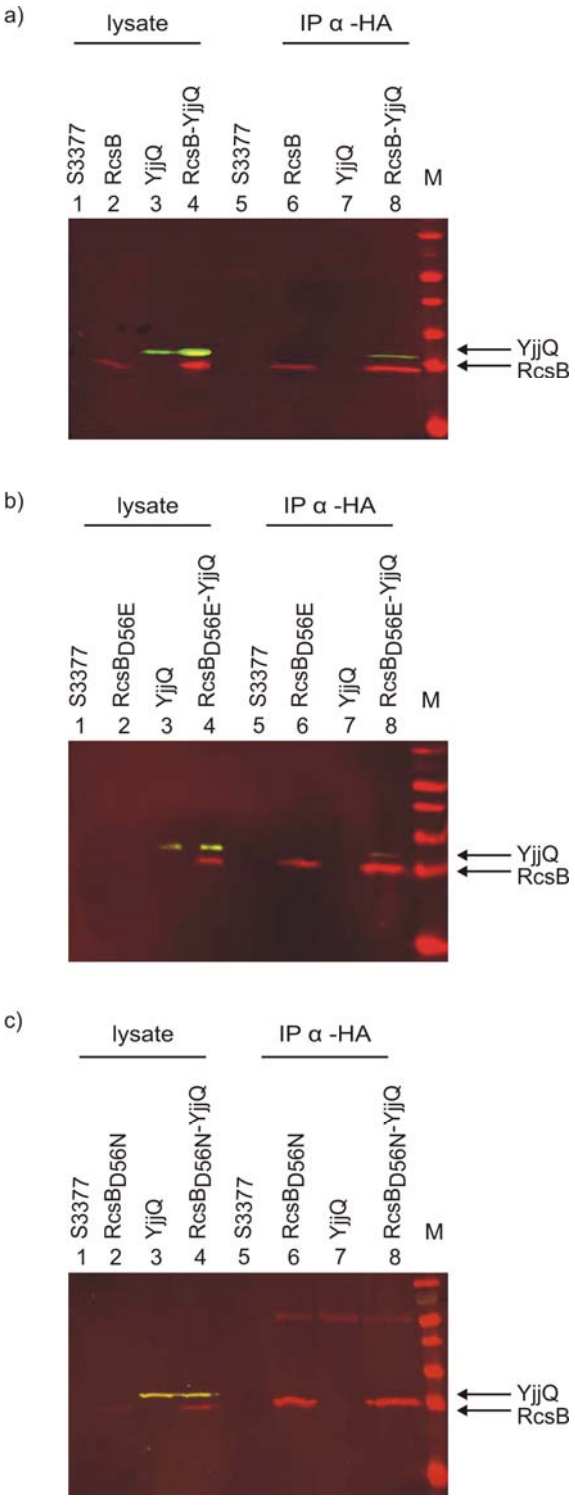


Fig 9. Interaction of YjjQ with RcsB, $RcsB_{D56E}$, and $RcsB_{D56N}$ analyzed by co-immunoprecipitation. For co-immunoprecipitation cell lysates were prepared of transformants of *E .coli* strain S3377 (Δ*rcsB*, Δ*yjjP-yjjQ-bglJ*) with plasmids expressing C-terminal tagged RcsB-HA and YjjQ-FLAG. Tested were the non-transformed strain expressing neither of the proteins and transformants expressing RcsB-HA or YjjQ alone, are both proteins. Cultures were grown in LB with antibiotics and protein expression was induced with 1mM IPTG for 2hrs at 37°C. 200µg of the total protein from the lysates were immunoprecipitated with Rabbit-anti-HA IgG antibody in conjunction with protein-A sepharose. The precipitates and the lysate were separated on a 12% SDS gel and blotted for Western analysis. The Western blot was analyzed using rat-anti HA and anti-rat alexaflour@680 antibodies (red color) for HA tagged proteins. Mouse-anti-FLAG and anti-mouse alexaflour@800 antibodies (green color) were used for FLAG tagged proteins. The blot was developed using an Odyssey scanner at 700 and 800nm channels. Co-Immunoprecipitation of YjjQ-FLAG (pKERV6) with (a) RcsB-HA (pKEAP38), (b) $RcsB^{D56E}$-HA (pKEAP43) and (c) $RcsB^{D56N}$-HA (pKEAP44).RcsB and YjjQ protein are indicated by an arrow.

2. RcsB-BglJ heterodimer binding to the *bgl* regulatory region

A transposon-mutagenesis screen for identifying factors which are required for activation of the *bgl* operon by BglJ identified the response regulator RcsB as a cofactor (Paukner, 2007). Further it was shown by two-hybrid analysis that RcsB and BglJ form heterodimers (unpublished data of the lab). Heterodimerization of RcsB and BglJ was substantiated in this thesis by co-immunoprecipitation analysis (Results chapter 1). As BglJ requires RcsB for activation (or rather de-repression) of the *bgl* operon, RcsB/BglJ heterodimers may bind to the *bgl* regulatory region and prevents repression of *bgl* by H-NS. This hypothesis is further supported by the identification of sequence motif which is very similar to one half-site of the consensus binding sequence of RcsA/RcsB heterodimers (Paukner, 2007) (and see below). The aim of the experiments described in this chapter was to characterize whether RcsB/BglJ heterodimers bind to the *bgl* regulatory region.

2.1 Linker insertion mutants of a putative RcsB/BglJ binding site in the *bgl* regulatory region

The response regulator RcsB regulates transcription by binding as a homodimer or as a RcsB-RcsA heterodimer (Stout and Gottesman, 1990) and see introduction). Consensus binding sequences for RcsB homodimers and RcsB-RcsA heterodimers were determined from various mutagenesis experiments in enterobacteriaceae family members (Ebel et al., 1997; Wehland et al., 1999). The RcsAB heterodimer binds to a specific sequence “TaAGaatatTCctA” called RcsAB box is located 70 to 100 base pairs upstream to the transcriptional start site. The RcsB homodimer binding sequence “GAAgaAtAACctgC**”** is located immediately upstream to the -35 regions, requiring interaction with RNA polymerase to stabilize the binding (Wehland and Bernhard, 2000; Sturny et al., 2003). Interestingly, Paukner, A. identified a sequence motif in the upstream of *bgl* regulatory region matching half of the RcsAB consensus sequence. The motif maps between positions -90 to -96bp upstream of transcription start site (Fig 10). This motif may represent a binding site of RcsB-BglJ heterodimer in the *bgl* regulatory region.

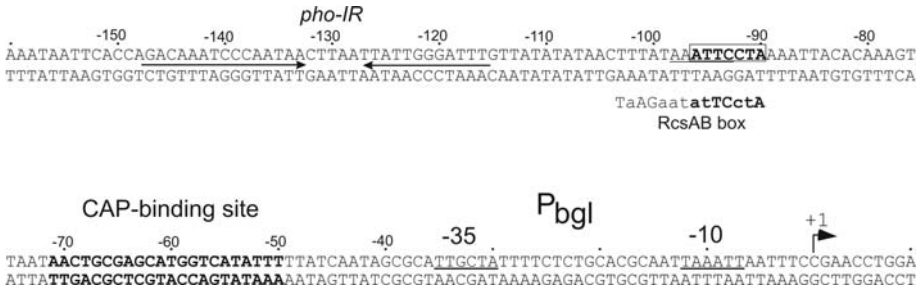


Fig 10. Putative RcsB/BglJ heterodimer binding site in *bgl* regulatory region. Schematic representation of *bgl* promoter region. The *bgl* promoter -10 and -35 sequence motifs are underlined. The CAP binding site is marked in bold. The transcription start site is indicated by an arrow with +1 number. Inverted arrows denote *bgl* operon transcriptional terminator t1 and inverted repeat *pho-IR*, respectively. The half matching consensus sequence (putative RcsB/BglJ box) is marked in bold. The RcsAB box consensus sequence (Wehland M et.al. 2000) is given below the putative RcsB-BglJ binding site.

The relevance of this putative RcsB-BglJ binding site was tested using a collection of plasmids carrying linker insertions in the upstream *bgl* regulatory region. With this collection of plasmids it was tested which linker insertions within the *bgl* upstream regulatory region abrogates activation of the *bgl* promoter by RcsB-BglJ. To measure the *bgl* promoter activity, the plasmids carry a *lacZ* reporter gene fused 3' to the first gene of the operon, *bglG* (Caramel and Schnetz, 1998). All plasmids with linker insertions carry in addition a single base-pair exchange within the regulatory region (creating an EcoRI-site used for construction of the linker insertion mutants). This mutation does not affect repression of *bgl* by H-NS (Caramel and Schnetz, 1998), and see below). The 6 bp MunI linker insertion map at different positions within the putative binding site, and also between the putative binding site and the promoter region, and upstream of the putative binding site (Fig 11). The *bgl* promoter activity was analyzed by β-galactosidase assay in strain S541 (Δ*bgl* Δ*lacZ*) and a derivative of a strain which constitutively expresses BglJ (strain S3910). As expected, the β-galactosidase activity of the control plasmid (pFMAC20) was low in the wild-type, and the activity was approximately 88 fold higher in the strain which constitutively expresses BglJ (Fig11). In the *rcsB* (strain S3912) background constitutive expression of BglJ caused no activation confirming that activation of *bgl* by BglJ requires RcsB (Fig11). The β-galactosidase activity directed by plasmids with MunI linker insertion was similar to the control plasmid when analyzed in the wild type strain (Fig11). In strain S3910 constitutively expressing BglJ, β-galactosidase expression directed by

plasmids carrying MunI linker insertion at positions -80, -88, -97, -99, -101, -103 and -123 were similar to the wild type strain (Fig11). These results demonstrate that mutations within the putative binding site and between the putative biding site and the promoter region prevent the activation by BglJ. In addition, a linker insertion upstream of the binding site at position -123 prevents activation by BglJ. These results suggest that BglJ-RcsB heterodimer bind to the putative RcsB-BglJ binding site within the *bgl* regulatory region.

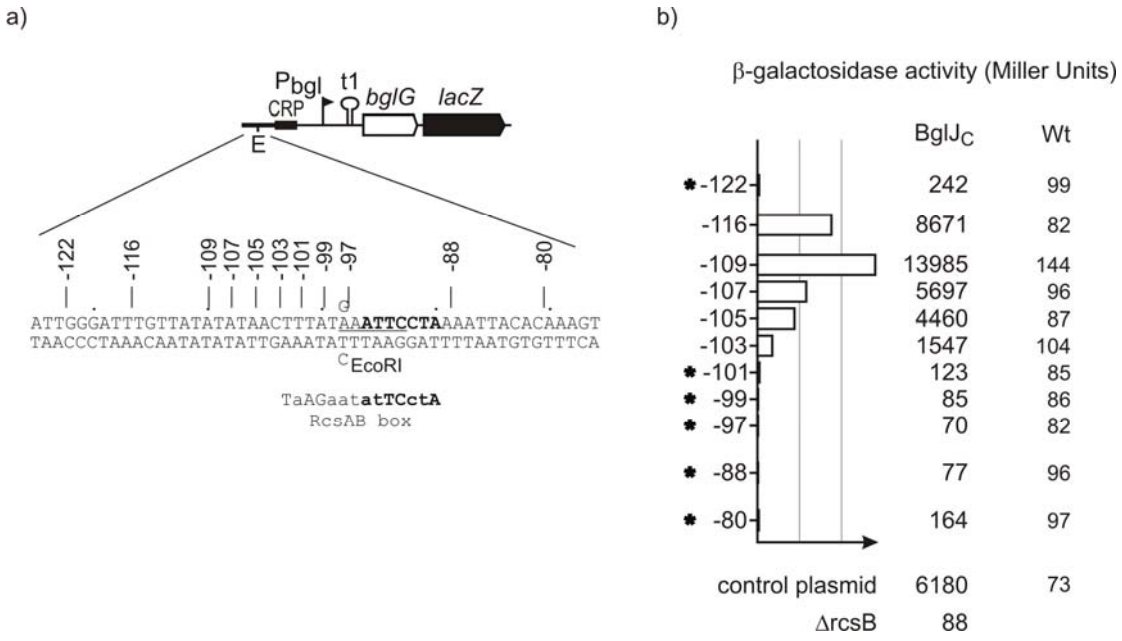


Fig 11. Mapping of a putative RcsB/BglJ binding site by linker insertion mutants in the *bgl* regulatory region. a) Schematic representation of plasmid constructs carrying the *bgl* regulatory region including the *bglG* gene followed by a *lacZ* reporter fusion. The CRP binding site, *bgl* terminator t1, bglG gene, and *lacZ* are indicated. The sequence matching half of the RcsAB consensus sequence is underlined and the RcsAB consensus sequence is given below. The vertical lines with the numbers represent the positions where 6 bp MunI linker were inserted. **b)** The β-galactosidase activity obtained from the plasmids expressed in strain S541 (Δ*bgl* Δ*lacZ*) and strain S3910 constitutively expressing BglJ. The control plasmid (pFMAC20) carries the native *bgl* regulatory region with a single base pair exchange creating an EcoRI site and the β-galactosidase activity obtained in rcsB deletion (S3912 (ΔrcsB::specR yjjQ/bglJ-Y6::mTn10cm) is also shown. The asterisk highlights linker insertion mutants which are not activated by RcsB/BglJ (assayperformed by Kathleen plumber, Lab technician).

2.2 Bacterial one-hybrid system for DNA-binding specificity

To study the RcsB/BglJ heterodimer binding to the putative binding site within the *bgl* regulatory region, I adapted a bacterial-two hybrid system for determining the DNA binding specificity of transcription factors created by Ann Hochschild (Dove and Hochschild, 2004; Dove, 2003; Dove et al., 1997; Hochschild and Dove, 1998).

In this approach, one of the protein domains to be tested (the bait) is fused to a sequence specific DNA-binding protein, and the other protein under investigation (prey) is fused to a subunit of the bacterial RNA polymerase (RNAP). The bacteriophage λ (λcI) is used as the DNA-binding protein, whereas the α subunit is used for fusion to RNA polymerase. Compatible plasmids expressing λcI and α subunit fusion protein are introduced into a suitable strain of *E. coli,* which contains a test promoter that drives the expression of a linked reporter gene. The test promoter consists of the *lac* core promoter and OL2 binding sites for λcI (Dove and Hochschild, 2004). The *lacZ* gene used as a reporter, whose activity can be easily measures by a colorimetric β-galactosidase assay. When induced, expression of the λcI fusion protein gene results in the binding of the corresponding λcI fusion protein to OL2. The expression of α fusion gene leads to the assembly of the resulting α fusion protein into RNA polymerase. Interaction between the DNA-bound λcI fusion protein and the assembled α fusion protein stabilizes the binding of RNAP to the test promoter, thus activating transcription of the reporter gene *lacZ* (Fig12).

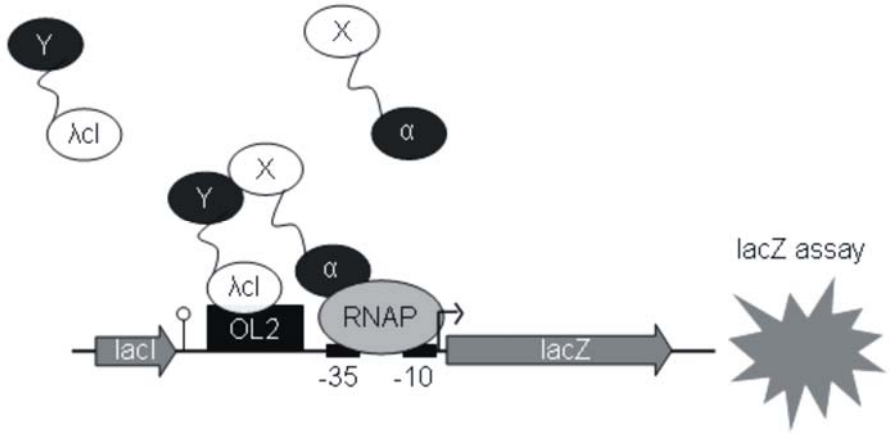


Fig 12. Principle of Ann Hochshild`s Bacterial Two-Hybrid system. Contact between the two protein domains X and Y are fused respectively to the α- N-terminal domain and to λcI activates the transcription from the lacZ reporter gene. The λcI binding site (OL2) positioned 62bp upstream from the transcription start site. The -10 and -35 regions are depicted in black boxes. The lac promoter drives the expression of *lacZ* reporter gene which can be measured by lacZ assay was indicated.

I utilized the system and modified it to a bacterial-one hybrid system for determining the DNA-binding specificity similar to bacterial one-hybrid system shown by (Meng et al., 2005). In this system the transcription factor RcsB was fused to α subunit of RNA polymerase and the interacting partner BglJ was over expressed

through a plasmid. The system was cloned with BglJ fusion to α subunit of RNA polymerase vice versa also. The restriction sites in the reporter were modified to replace the OL2 binding site containing DNA fragment with *bgl* regulatory fragment. In theory, upon induction, α fusion RcsB protein forms heterodimer with BglJ protein, and the heterodimer binds to the *bgl* regulatory region. This should lead to the recruitment and stabilized binding of RNA polymerase to the promoter and thus activation of the *lacZ* reporter gene (Fig 13).

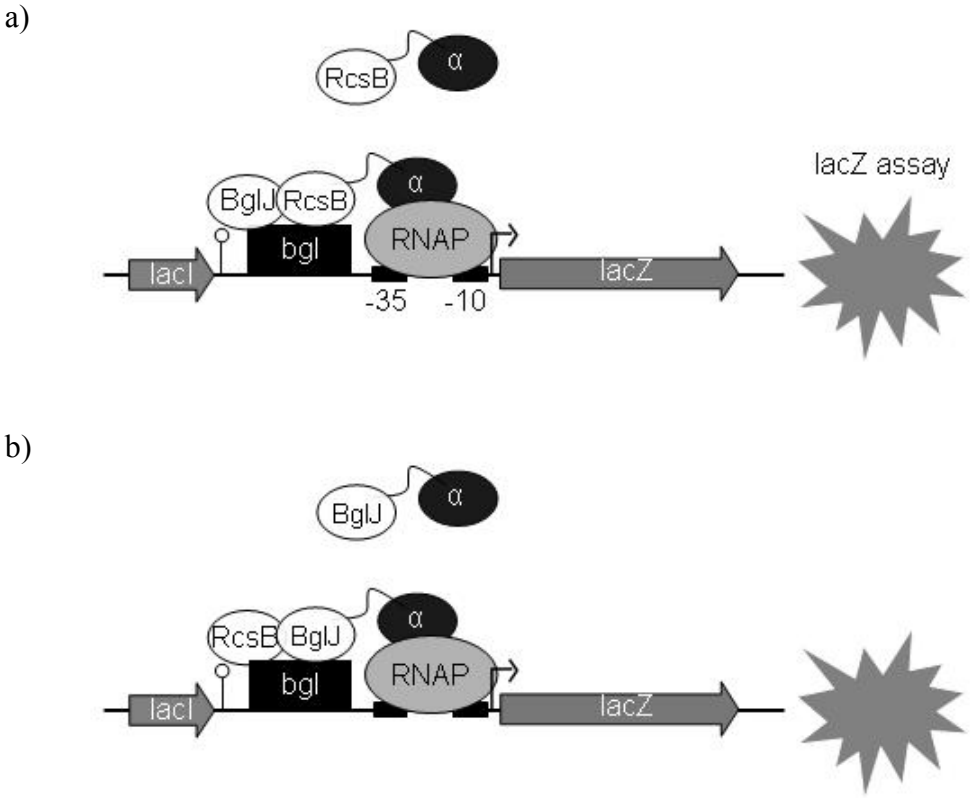


Fig 13. Principle of Bacterial one-Hybrid system. (a) The transcription factor (RcsB) was fused to the α- N-terminal domain and BglJ was over expressed through a plasmid. **(b)** The transcription factor (BglJ) was fused to the α- N-terminal domain and RcsB was over expressed through a plasmid The λcI binding site (OL2) positioned 62bp upstream from the transcription start site was replaced by *bgl* regulatory region RcsB/BglJ heterodimer as expected to be bind to the bgl regulatory region leads to RNAP recruitment. This activates the transcription from the lacZ reporter gene.. The -10 and -35 regions are depicted in black boxes. The lac promoter drives the expression of *lacZ* reporter gene which can be measured by lacZ assay was indicated.

2.2.1 Testing bacterial one-hybrid system

To test the bacterial one-hybrid system, I constructed a plasmid (pKERV45) carrying a test promoter, *Plac* which consists of a λ operator (OL2) positioned 62 base pairs upstream from the transcription start site (Fig 14a). The plasmid also carries a

modified restriction site for *attB* site integration in to the *E. coli* chromosome. The upstream of the promoter also maps *lacI*^{q} gene. The OL2 fragment was positioned between two restriction sites for replacement purposes. The plasmid was integrated into *E. coli* strain (S4911) carrying a deletion of *bgl* and *lacZ* genes. The control plasmids (see Materials and methods Table 3) were transformed into *E. coli* (S4911) and LacZ assay was performed (Fig 14b). The positive control plasmids expressing λcI-β$^{831\text{-}1057}$ fusion with α-σ70^{D581G,} gave approximately 3 fold higher activity than the negative controls. The negative control plasmids were expressing either α fusion or λcI fusions, which are not able to interact with RNA polymerase.

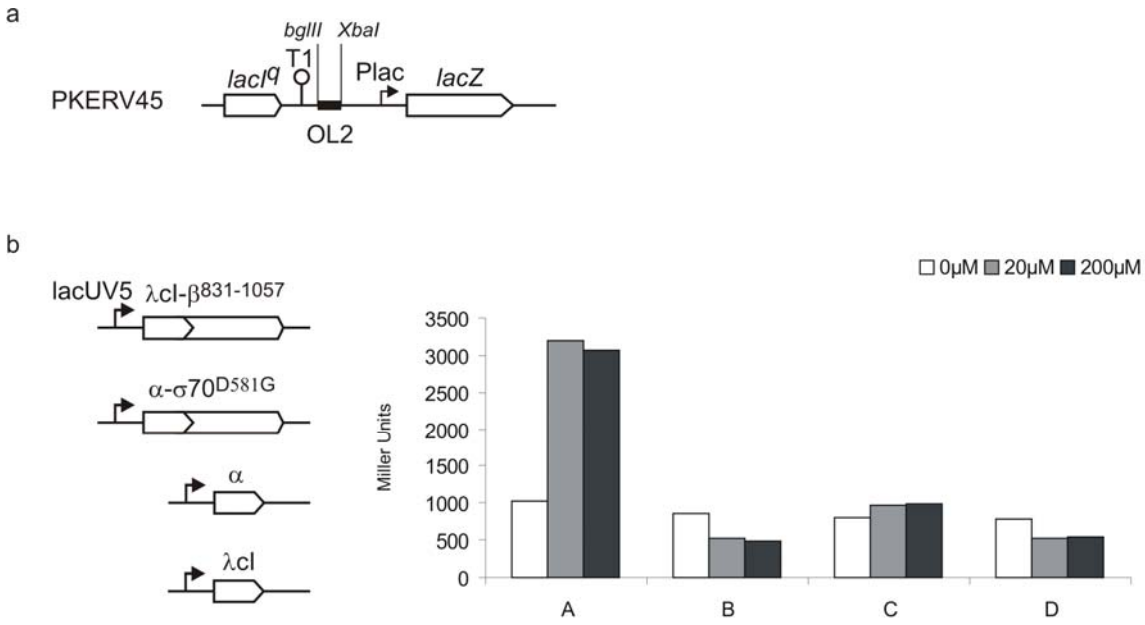


Fig 14. Testing bacterial one-hybrid system. (a) The plasmid (pKERV45) carrying the OL2 binding site for λcI protein was integrated in to *E. coli* (S541). The Plac promoter, lacI^{q} gene and terminator (T1) and lacZ reporter gene are indicated. The transcription start site is mentioned by an arrow. The OL2 flanking restriction site bglII and XbaI are mentioned (b) The lacZ assay of positive control (A) with plasmids expressing λcI-β$^{831\text{-}1057}$ and α-σ70^{D581G} and the negative controls (B, C, D) with λcI-β$^{831\text{-}1057}$, α fusion, λcI with λcI-β$^{831\text{-}1057}$and λcI with α fusion. The ON cultures were induced with 0μM, 20μM and 200μM IPTG and mid-exponential cultures at OD_{600} =0.5 were used for the assay.

2.2.2 Analysis of putative binding site

To check the RcsB/BglJ heterodimer binding in the *bgl* regulatory region, I replaced the OL2 binding sites with *bgl* regulatory region carrying the putative RcsB/BglJ binding site. Since the orientation of heterodimer binding is unknown, the plasmids were constructed carrying the putative box in both direct and inverse complement orientations (Fig 15).

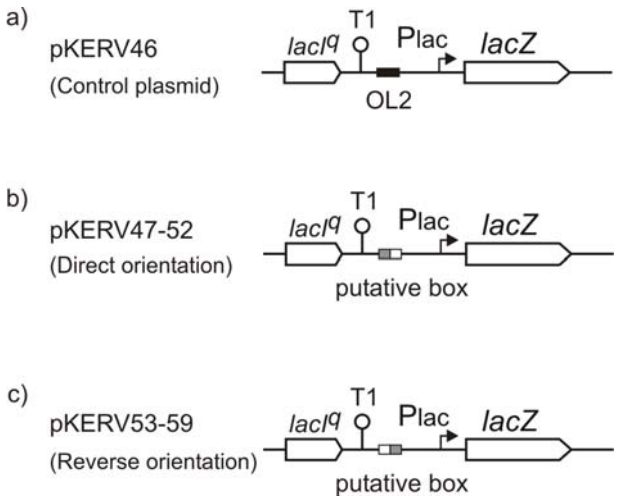


Fig 15. Plasmids and constructs used in Bacterial one-Hybrid system. The plasmids carry a *lacI*^{q} gene and terminator (T1) and *Plac* promoter with a *lacZ* reporter fusion. **a)** The control plasmid (pKERV46) carrys OL2 fragment positioned 62 upstream from the transcription start site. **b)** The plasmids (pkERV47 to pKERV52) carrys of *bgl* regulatory fragment from position -108 to -79 (direct orientation) **c)** The plasmids (pkERV53 to pKERV59)consist of *bgl* regulatory fragment from position -120 to -87 (reverse orientation).

Briefly, the plasmid pKERV47 consists of *bgl* fragment from position -108 to -87 in a direct orientation and plasmids pKERV48 to pKERV52 were constructed with each plasmid carrying an additional 2 bp to the upstream region, to cover one helical turn (approximately 10.5 base pairs). Similarly the Plasmids, pKERV53 to pKERV59 were constructed with plasmids carrying *bgl* fragment in an inverse orientation from position -120 to -87 (pKERV47 to pKERV59 (Fig 16) (See materials and methods 6).

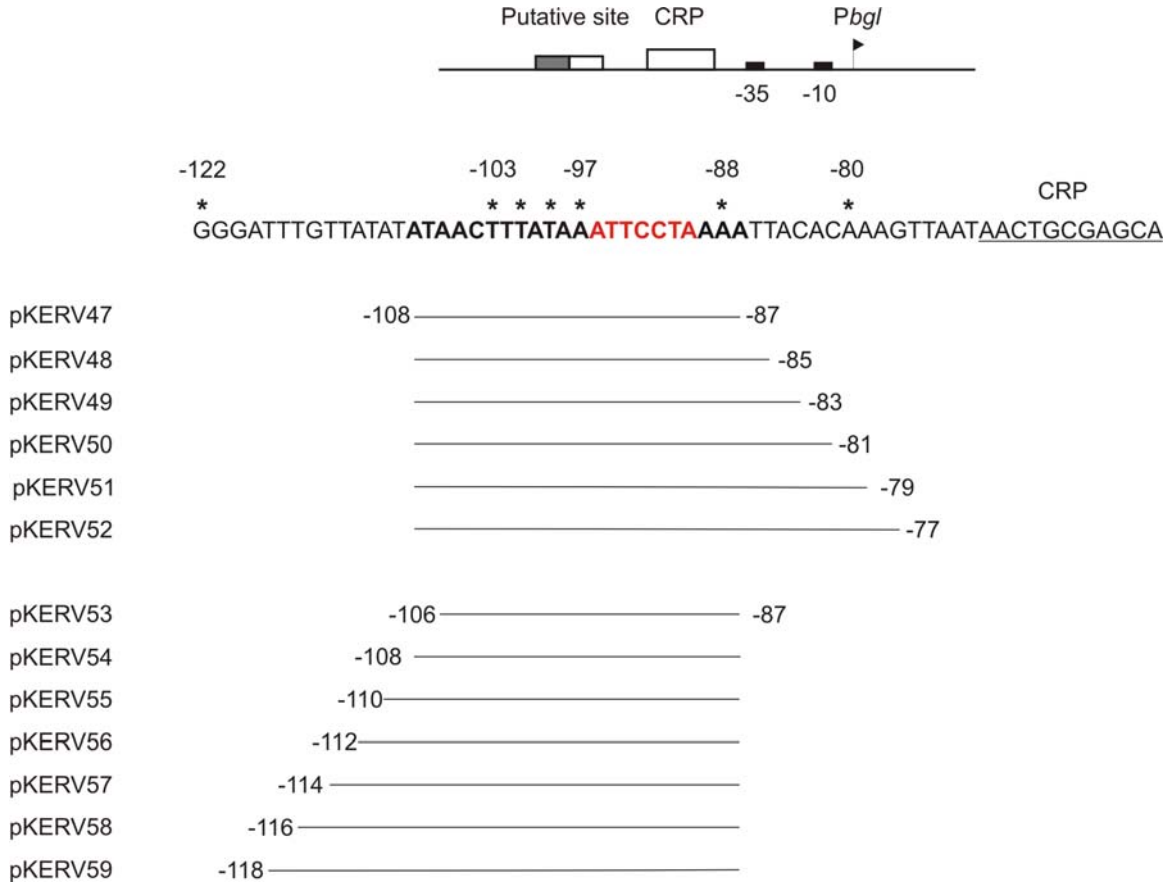


Fig 16. Plasmids and constructs used in Bacterial one-Hybrid system. In the scheme, the -10 and -35 promoter regions are shown as small black boxes and the transcription start site is shown by an arrow. Structure of the *bgl* regulatory region including the putative RcsB/BglJ box, shown as grey/white box. The white part represents the half matching RcsAB consensus sequence. The CRP binding sites is also indicated (top); Sequence of the *bgl* regulatory region including the putative RcsB/BglJ box sis shown. The numbers and asterisk indicate linker insertion mutants which are not activated by BglJ. All the plasmids carry a *lacI*^{q} gene and terminator (T1) and *Plac* promoter with a *lacZ* reporter fusion. The plasmids (pkERV47 to pKERV52) consist of *bgl* regulatory fragment from position -108 to -79 (direct orientation) and the plasmids (pkERV53 to pKERV59) carrys *bgl* regulatory fragment from position -120 to -87(inverse complement orientation).

The plasmids carrying RcsB/BglJ binding site (pKERV47 to pKERV59 (pSC101, cm ori) were transformed into *E. coli* strain S4160 which carries deletions of *rcsB* and *bglJ* genes. The three compatible plasmids expressing α-RcsB fusion protein (pKERV18), plasmid expressing BglJ (pKETS01) and plasmids carrying RcsB/BglJ putative site were co-transformed into *E. coli* (strain S4160). As a control the plasmid which carries OL2 binding site (pKERV46) used. The β-galactosidase activity from the empty strain expressing *bgl* fragment carrying plasmids and strain expressing α fusion RcsB and BglJ are shown in figure 17.

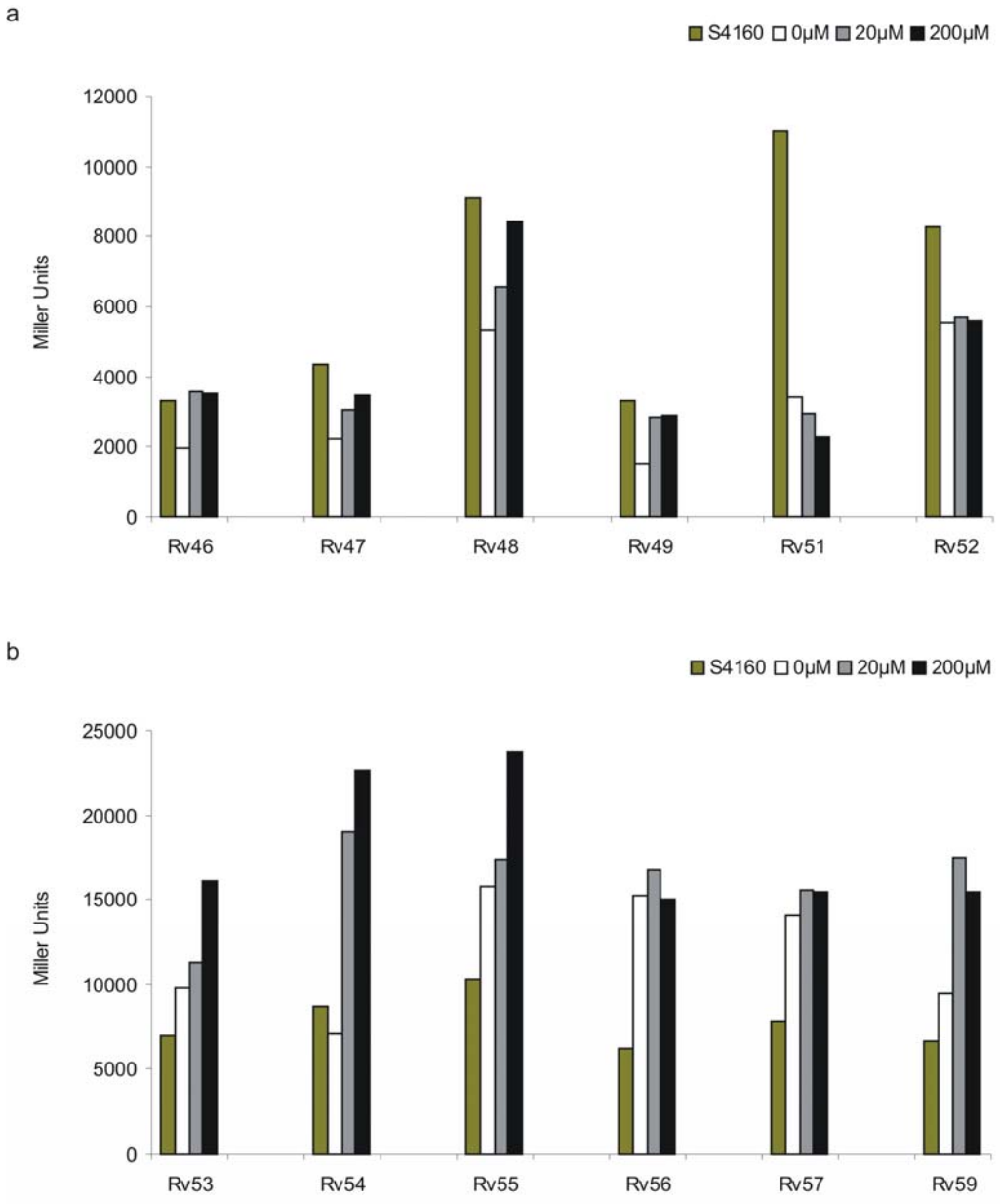


Fig 17. β-galactosidase assay from Strain S4160. The plasmids carrying *bgl* regulatory fragment (pKERV47 to pKERV59), plasmids expressing α fusion RcsB (pKERV18) and plasmid expressing BglJ (pKETS01) were transformed into S4160. Only transformant with empty plasmid (caryying Ol2 binding site vector (pKERV46) used as control. The strains were grown in LB medium with 0μM, 20μM and 200mM IPTG at 37ºC and 0.5 OD600 cells were used for β-galactosidase activity. Plasmid pKERV46 to pKERV52 carries *bgl* regulatory fragments in direct orientation (-108 to -77) (a). Similarly plasmids pKERV53 to pKERV59 carries *bgl* regulatory fragments in inverse orientation (-120 to -87) (b) (See Materials and methods 11).

The plasmids which carry the putative RcsB/BglJ binding site in direct orientation (-108 to -77) (pKERV47 to pKERV52) showed similar or higher activity in empty strain than the strain expressing RcsB and BglJ (Fig 17a). The plasmids carrying the

putative RcsB/BglJ binding site in inverse orientation (-120 to -87) (pKERV53 to pKERV59) showed 2 fold higher activity than the empty strain (Fig 17b).

To test weather the two fold increase in activity was due to the effect of RcsB and BglJ proteins. The Selected plasmids (activity > 2 fold) were co-transformed into *E. coli* strain S4160 with the empty vector (α vector) and the β-galactosidase activity was measured (Fig 18). The activity obtained from strain expressing empty vector were similar to strain expressing RcsB and BglJ. The 2 fold difference which was observed earlier in the empty strain (Fig 17b) was lost. We do not understand the loss of activity in the control. The basal level of expression in empty strain is too high and due to this basal level expression this system cannot be used to estimate the effect of the RcsB and BglJ protein in the induced conditions.

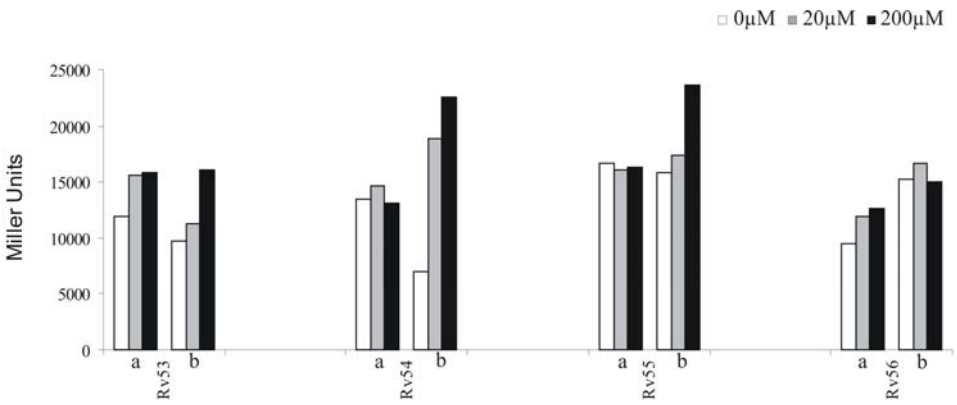


Fig 18. The β-galactosidase activity in S4160 strain with empty vector (pBRα). Each plasmid grown in strain with empty vector (a) and strain expressing RcsB and BglJ (b) were compared. The strain was grown in LB medium with 0μM, 20μM and 200mM IPTG at 37ºC and 0.5 OD600 cells were used for β-galactosidase activity.

2.3 Protein purification and Electrophoretic Mobility Shift Assay (EMSA)

The experiments described above were approaches to detect *in vivo* binding of RcsB-BglJ to the putative RcsB-BglJ binding site in the *bgl* regulatory region. In parallel, binding of RcsB-BglJ heterodimer to the putative binding site was to be characterized by electrophoretic mobility shift assays (EMSA) *in vitro*.

2.3.1 Expression of Epitope tagged proteins

Since the expression levels of C-terminal HA tagged RcsB mutants were weaker (See result 1) and other C-terminal tagged vectors caused problem in expression of RcsB proteins (unpublished lab data), I decided to construct an N-terminal fusion proteins. I used N-term strep-tagged fusion proteins of RcsB and BglJ protein for the purification. The *rcsB* and *bglJ* genes, respectively, were cloned into plasmids pKERV29 and pKERV30, which carry the inducible *Ptac* promoter followed by a sequence encoding the Strep-tag (WSHPQFEK, (Schmidt and Skerra, 1994; Schmidt et al., 1996; Korndorfer and Skerra, 2002). For efficient translation of the Strep-tagged proteins the plasmids carry the strong Shine-Dalgarno sequence derived from phage T7 gene10. Upstream of the *tac* promoter maps the *lacI*^{q} gene. Low and high copy variants of these vectors were cloned (Fig 19). In addition, the *rcsB* mutants D56E and D56N as well as *yjjQ* were also cloned into these plasmid (Fig 19). The expression and solubility of the Strep-tagged RcsB, $RcsB_{D56E}$, $RcsB_{D56N}$ and BglJ proteins encoded by these plasmids was tested by Western blots (Fig 20 and 21).

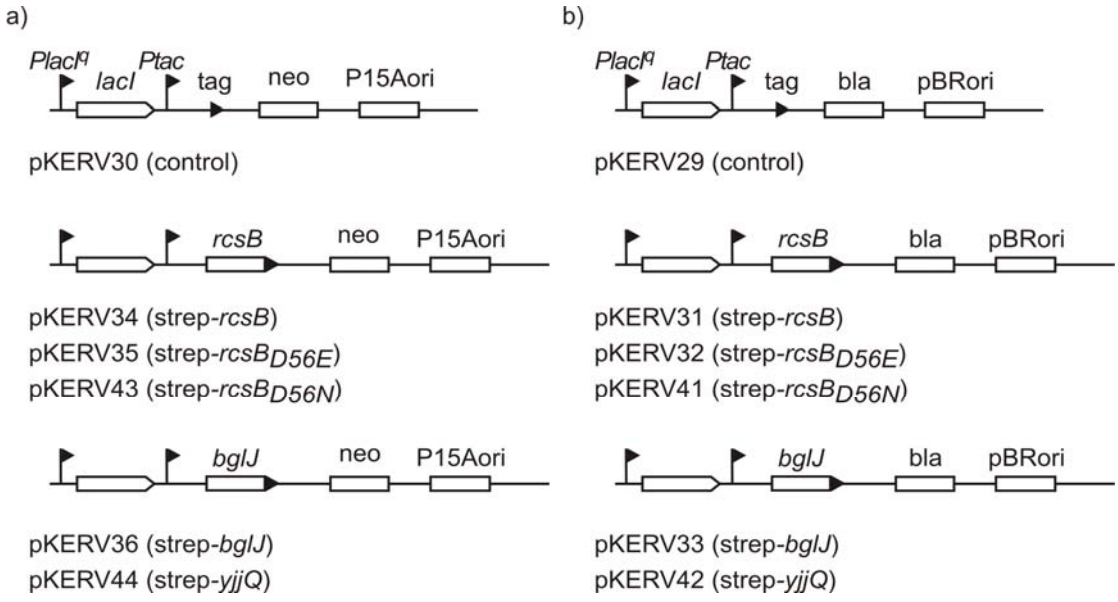


Fig 19. Plasmids for expression of N-terminally Strep-tagged RcsB, $RcsB_{D56E}$, $RcsB_{D56N}$, BglJ and YjjQ proteins. Schematic representation of plasmids encoding N-terminally Strep-tagged BglJ,YjjQ and RcsB variants (a,b). The low copy plasmids (a) carry a p15A origin of replicartion and kanamycin resistance marker (*neo*). The high copy plasmids (b) carry a pMB1 origin (a high copy variant) and ampicillin resistance marker (bla). The *bgl* and *rcsB* genes were cloned under an IPTG inducible *tac* promoter. All the genes were constructed with a strep fusion tag at the 5`end. Plasmids pKERV29 and pKERV30 are the parent plasmids which carry the strep-tag fused to the gene10 Shine Dalgarno sequence followed by NcoI and XbaI sites for cloning.

The plasmids were transformed into strain S3377 (Δ*yjjP-yjjQ-bglJ*), Δ*rcsB*) carrying deletions of *rcsB* and *bglJ* genes. The cells were grown to OD_{600}=0.3 and protein synthesis was induced with 1mM IPTG for 2 hours at 37ºC ($RcsB_{D56E}$ was grown at 28ºC for better protein production). The expression and solubility of the induced cultures were checked on coomassie gels and confirmed by western blotting. The expression of the strep-tagged RcsB and its mutants was better when encoded by high copy plasmids (Fig 20c) than by low copy variants (Fig 20a). But the solubility of the RcsB and its mutants were better in low copy plasmids (Fig 21a) than a high copy variant (Fig 21.c). The expression of the Strep-tagged BglJ protein showed no significant difference between a low copy (Fig 20b) and a high copy variant (Fig 20d). The solubility of Strep-tagged BglJ protein was marginally better when encoded by the high copy plasmid than the low copy plasmid (Fig 21 d and b), but expression of BglJ was weaker than expression of RcsB. Expression of YjjQ expression was marginally higher when encoded by the high copy than by the low copy plasmid (Fig 21b and d). No significant difference in the level of protein production was seen in un induced and induced cultures (Fig 20 b and d).

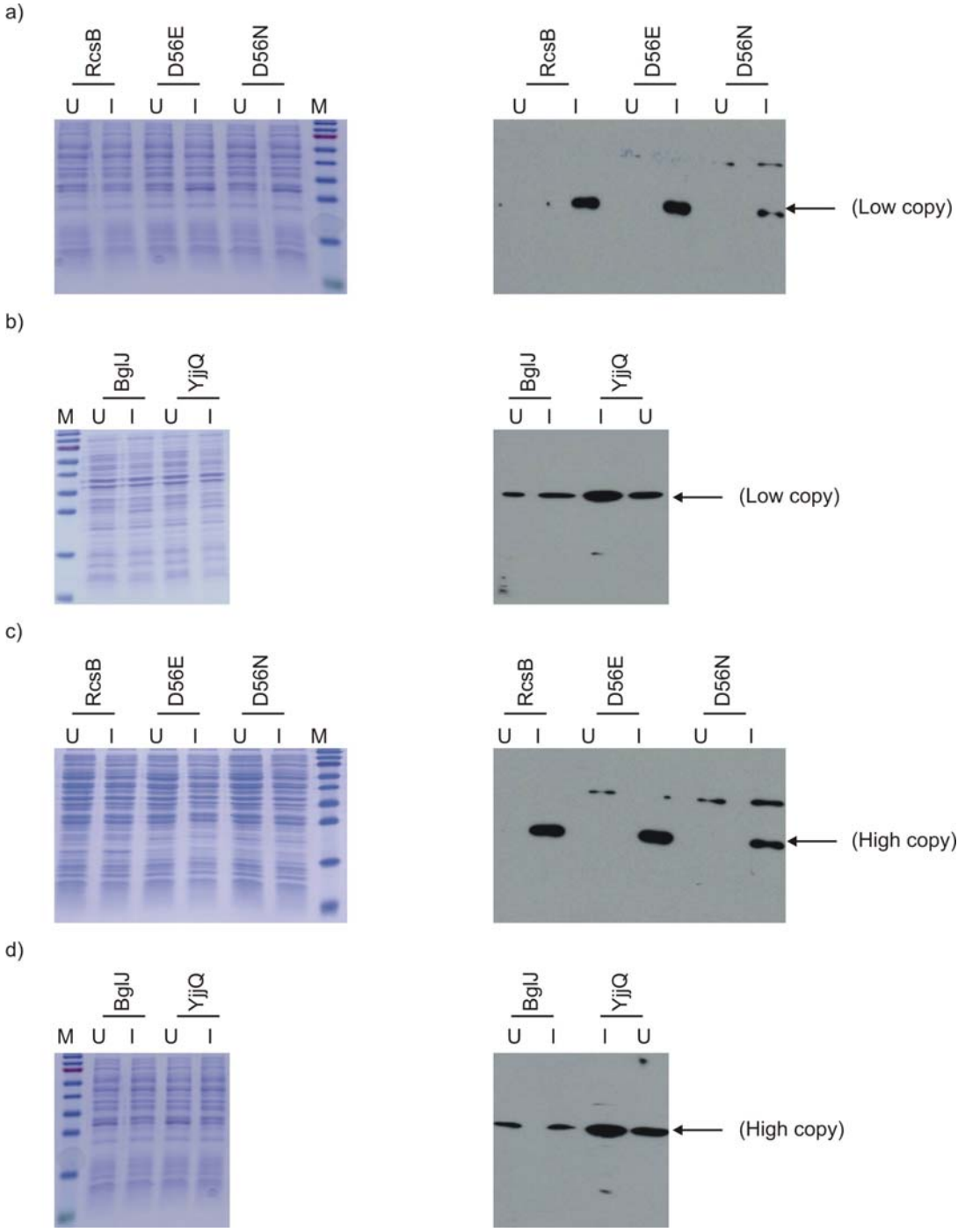


Fig 20. Expression of N-terminal strep tagged RcsB, $RcsB_{D56E}$, $RcsB_{D56N}$, BglJ and YjjQ from high and low copy variants. High and low copy plasmids encoding Strep-tagged RcsB, BglJ and YjjQ were used to transform into *E. coli* strain S3377 (Δ*yjjP-yjjQ-bglJ*) and Δ*rcsB* genes. Cultures were grown at 37°C and protein expression was induced with 1mM IPTG for 2 hours. The expression of the protein was analyzed from the uninduec (U) and induced cells (I). The cells were resolved on 15% SDS-PAGE and analyzed by western blotting using anti-Strep-HRP conjugated antibody. Each lane was loaded with the equivalent of $0.05OD_{600}$ cells. Low copy plasmids (a, b) RcsB (pKERV34), RcsB-D56E (pKERV35), RcsB-D56N (pKERV43), BglJ (pKERV36), YjjQ (pKERV44). High copy plasmids (c,d) RcsB (pKERV31), RcsB-D56E (pKERV32), RcsB-D56N (pKERV41), BglJ (pKERV33), and YjjQ (pKERV42). The ~25 kDa RcsB, ~27 kDa BglJ and ~28 kDa YjjQ Strep-tagged proteins are indicated by an arrow.

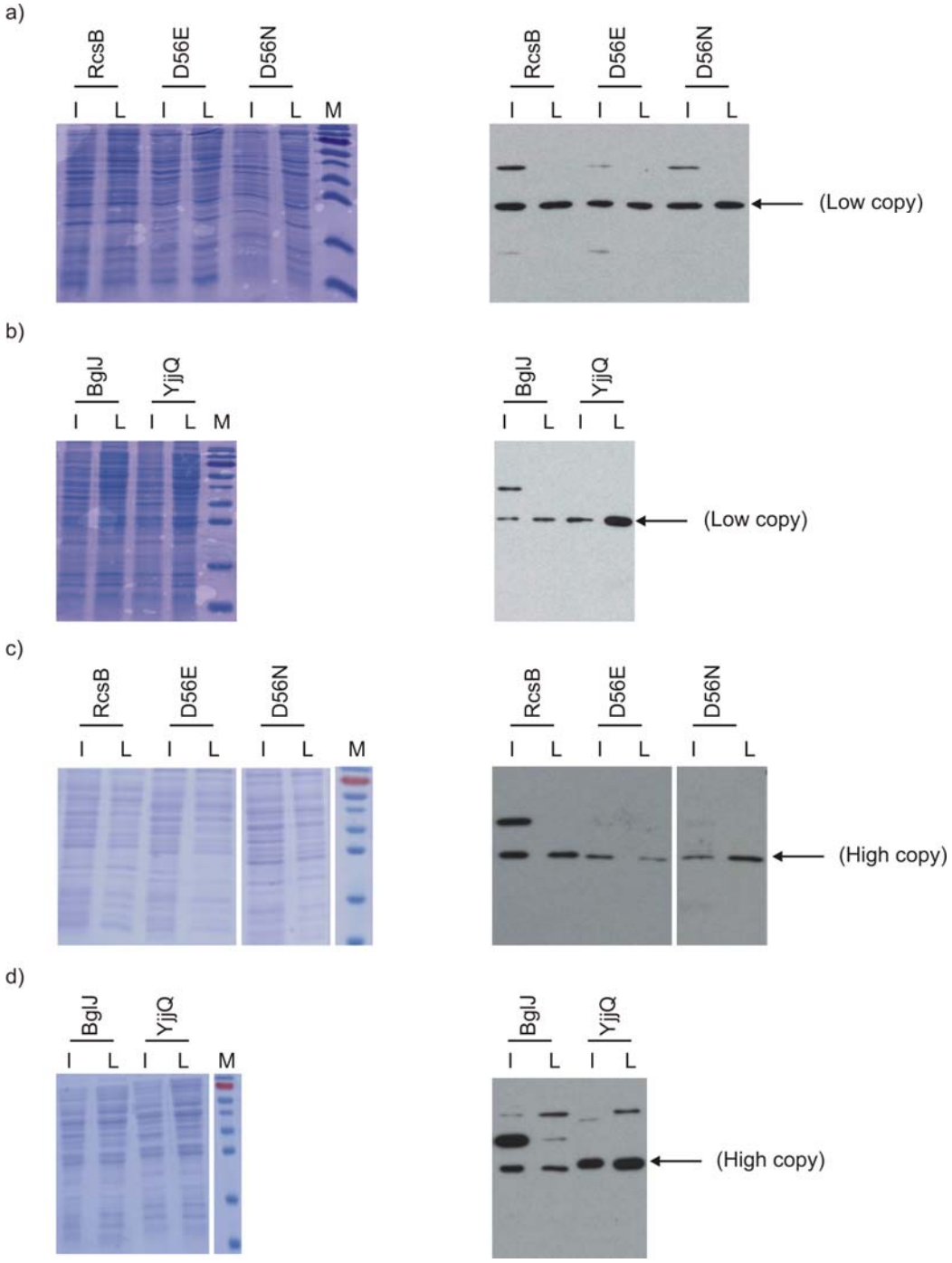


Fig 21. Expression for solubility of N-terminal strep tagged RcsB, $RcsB_{D56E}$, $RcsB_{D56N}$, BglJ and YjjQ from high and low copy variants. High and low copy plasmids encoding Strep-tagged RcsB, BglJ and YjjQ were used to transform into *E. coli* strain S3377 (Δ*yjjP-yjjQ-bglJ*) and Δ*rcsB* genes. Cultures were grown at 37°C and protein expression was induced with 1mM IPTG for 2 hours. The expression of the protein was analyzed from the induced cells (I). Cell free protein lysates (L) were prepared for checking the solubility of the protein. The cells were resolved on 15% SDS-PAGE and analyzed by western blotting using anti-Strep-HRP conjugated antibody. Each lane was loaded with the equivalent of $0.05OD_{600}$ cells. Low copy plasmids (a, b) RcsB (pKERV34), RcsB-D56E (pKERV35), RcsB-D56N (pKERV43), BglJ (pKERV36), YjjQ (pKERV44). High copy plasmids (c,d) RcsB (pKERV31), RcsB-D56E (pKERV32), RcsB-D56N (pKERV41), BglJ (pKERV33), and YjjQ (pKERV42). The ~25 kDa RcsB, ~27 kDa BglJ and ~28 kDa YjjQ Strep- tagged proteins are indicated by an arrow.

2.3.2 Strep-tag protein purification

Since the protein solubility levels of RcsB and its mutants were better in the low copy plasmids (See Fig 20 and 21), I used low copy variants of strep-tagged RcsB (pKERV34), $RcsB_{D56E}$ (pKERV35) and $RcsB_{D56N}$ (pKERV43) respectively for the protein purification. The plasmids were used to transform *E. coli* strain S3377 (Δ*yjjP-yjjQ-bglJ*), Δ*rcsB*). The cells were grown to OD_{600}=0.3 and protein synthesis was induced with 1mM IPTG for 2 hours at 37ºC ($RcsB_{D56E}$ was grown at 28ºC for better protein production). The cells harvested from 1 liter of IPTG induced culture were lysed, and the Strep-tagged RcsB wild-type and mutants proteins were purified using a Strep-Tactin® Superflow® Cartridge H-PR, (IBA, Germany) (see Material and Methods.15) using FPLC (Fig 22). The protein fractions were checked on 15% PAGE and the purest protein fractions were dialyzed. The Strep-tactin affinity column purified RcsB (fraction 21, 0.6mg/ml, buffer), $RcsB_{D56E}$ (fraction 20, 0.5mg/ml) and $RcsB_{D56N}$ (fraction 22, 0.4mg/ml) proteins (Fig26 to 29) were directly used for EMSA studies.

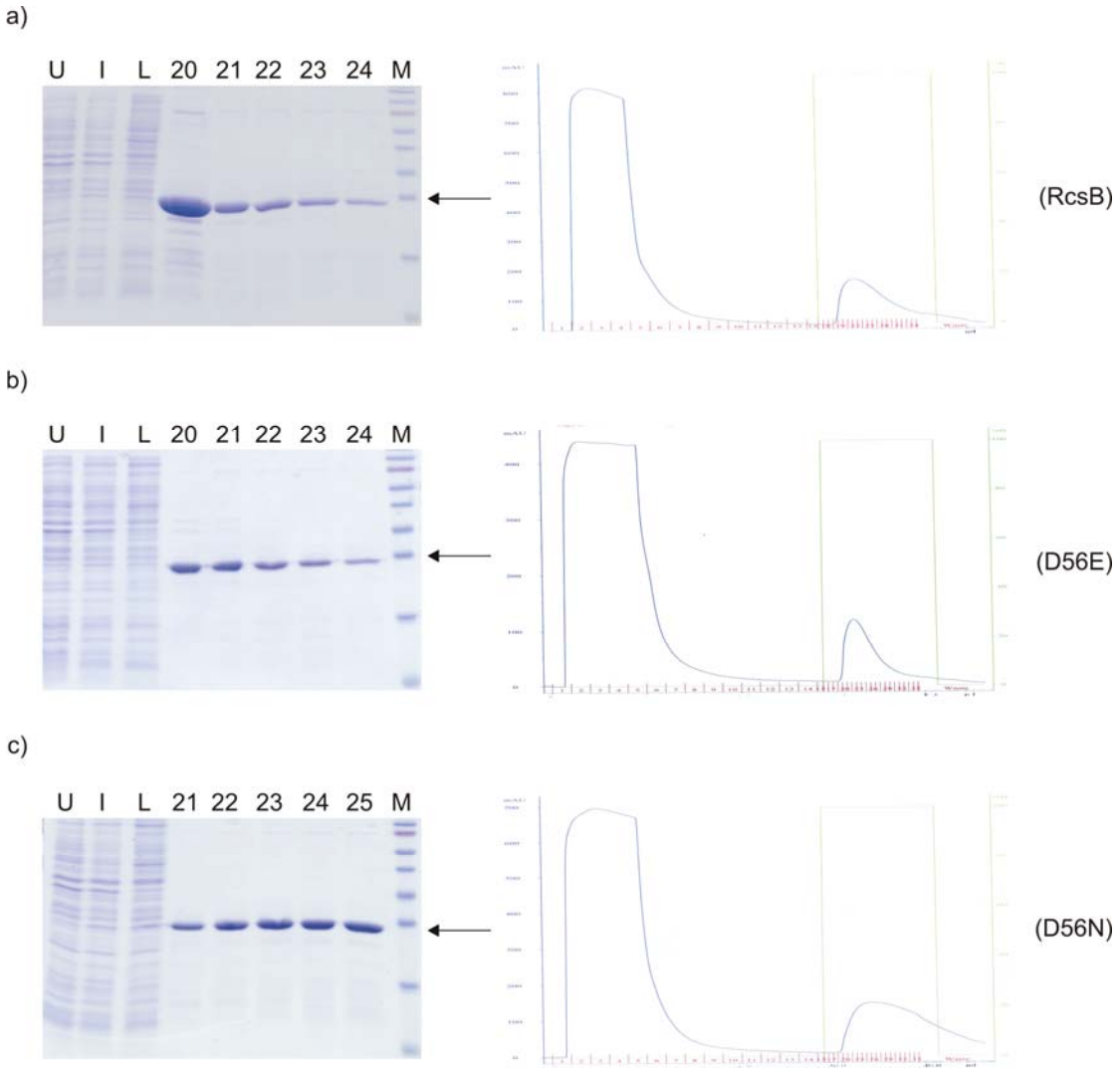


Fig 22. Strep-tactin affinity purification of N-terminally Strep-tagged RcsB, $RcsB_{D56E}$ and $RcsB_{D56N}$ proteins using ÄKTA-FPLC a) Purification of Strep-RcsB (encoded by pKERV34), b) $RcsB_{D56E}$ (pKERV35), and c) $RcsB_{D56N}$ (pKERV43). Left panel: Coomassie gel with aliquots equivalent to 0.05 OD_{600} cells of the uninduced (U) and induced (I) clutures, the lysate (L), and the 10 µl of fractions (numbers) collected after elution with Desthiobiotin. Right: Elution profile of the purification with a 1ml Strep-Tactin® Superflow® Cartridge H-PR (IBA, Germany) using an ÄKTA-FPLC (GE Healthcare Lifesciences, Freiburg). Indicated are the fractions and the UV 280nm. M is prestained protein marker (MBI fermentas). The ~ 25kDa Strep-tagged RcsB and mutant proteins are indicated by an arrows.

The purification of N-terminal strep-tagged BglJ protein using strep-tactin affinity column yielded very little protein (Fig 24). The purification could neither be achieved with the low copy plasmid nor with the high copy plasmid encoding Strep-BglJ. The expression of strep tagged BglJ fusion proteins was tested at different temperatures (28ºC and 37ºC) resulted in either reduced protein levels. The BglJ protein solubilization using mild non-ionic detergents like TritonX-100 and Nonidet P-40 (Sigma) did not result in high yield protein purification.

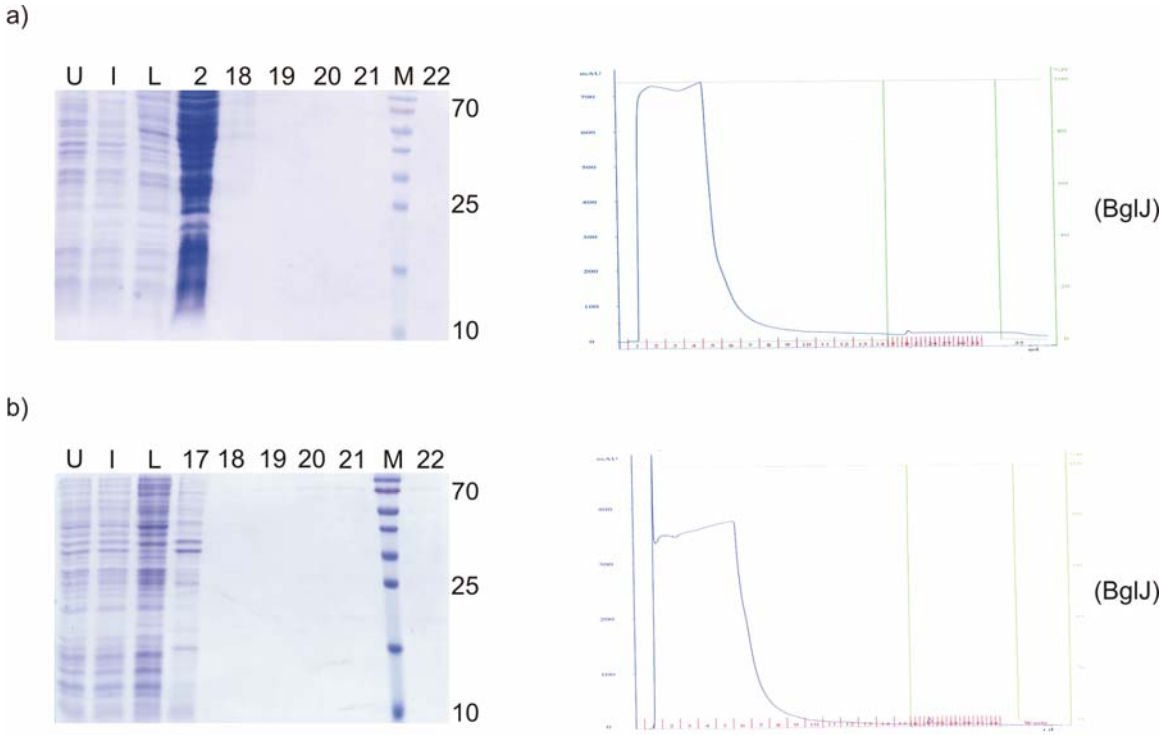


Fig 23. Strep-tactin affinity purification of Strep-BglJ protein using AKTA-FPLC. Purification of N-terminally strep-tagged BglJ protein encoded by low copy plasmid pKERV36 (a) and high copy plasmid pKERV33 (b) was unsuccessfull. Aliquots equivalent to 0.05 OD_{600} cells of uninduced (U), induced (I) cultures and of the lysate (L) were separated by SDS-PAGE along with 20 µl of fractions (numbers indicated) collected after elution with desthiobiotin. M: prestained marker (MBI Fermentas).

2.3.3 BglJ protein purification

Purification of N-terminally Strep-tagged BglJ was not successful. As C-terminally FLAG-tagged BglJ which was used in the co-immunoprecipitation experiments was soluble, BglJ-FLAG was purified using a FLAG affinity column. To purify the C-terminal FLAG tagged BglJ protein, strain S3377 (Δ(*yjjP-yjjQ-bglJ*), Δ*rcsB*) was transformed with plasmids pKERV10 (low copy) and pKERV14 (high copy) encoding C-terminally FLAG tagged BglJ. Expression of the *bglJ*-FLAG gene was induced with 1mM IPTG for 2 hours at 37ºC. Bacteria harvested from 1 liter of IPTG induced culture were lysed and purified using an FLAG affinity column (EZview Red ANTI-FLAG M2 affinity gel, Sigma). The eluted protein was dialyzed and the yield and purification was confirmed by SDS-PAGE followed by Coomassie staining and western blotting with mouse anti-FLAG/anti-mouse alexaflour® 680 antibodies (See materials and methods 13) (Fig 24). The partially purified protein was directly used for EMSA assays. The high copy variant expressing BglJ protein showed more

protein in insoluble fractions, (Fig 24.b lane p) suggesting over production of the BglJ protein might be accumulated as insoluble protein aggregate.

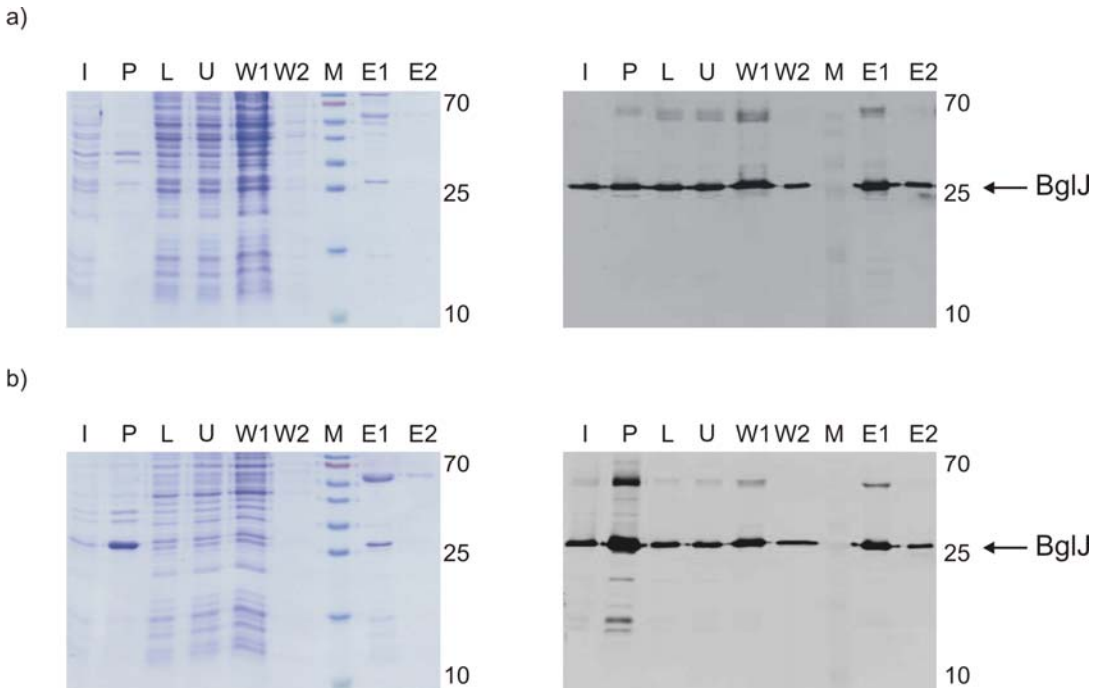


Fig 24. Affinity purification of BglJ-Flag protein. C-terminally FLAG tagged BglJ was purified from cells transformed with the low copy plasmid pKERV10 (a) or the high copy plasmid pKERV14 (b), which both code for BglJ-FLAG. Left panel: Coomassie gel with aliquots equivalent to 0.05 OD_{600} cells of the uninduced (U) and induced (I) clutures, the lysate (L), pellet (P) and the 10 µl of fractions (numbers) of wash (W) and elution (E) loaded. The ~27kDa BglJ protein was indicated by an arrow.

2.3.3 Electrophoretic mobility shift assay (EMSA)

To characterize whether RcsB/BglJ heterodimer binds to the *bgl* regulatory region by an *in vitro* assay, DNA electrophoretic mobility shift assays (EMSA) were performed. Briefly, a DNA fragment of the *bgl* regulatory region which encompasses the putative RcsB/BglJ binding site was incubated with increasing concentrations of purified Strep tagged RcsB, $RcsB_{D56E}$ and $RcsB_{D56N}$ proteins in the presence and absence of partially purified BglJ-FLAG protein. In addition, it was tested whether the H-NS protein affects binding of RcsB and RcsB-BglJ heterodimers. The protein-DNA complex was separated on 8% non-denaturing polyacrylamide gel and shifts were visualized by ethidium bromide staining. As controls a *lacZ* fragment which is not bound by RcsB and an *osmC* fragment which contains RcsB binding site were

included. The schematic presentations of the DNA fragments used in this assay are shown in Fig 25.

For the EMSA studies, a small 131bp *bgl* regulatory fragment from position -191 to -60 which encompasses the putative RcsB/BglJ binding site was used (Fig 25a). A larger *bgl* fragment from position -191 to +27 which encompasses the putative RcsB/BglJ binding site and core *bgl* promoter region was also used (Fig 29). A 241 bp fragment of the osmotically inducible *osmC* gene, which is repressed by H-NS (Bouvier et al., 1998) and positively regulated by RcsB (Sturny et al., 2003), (Davalos-Garcia et al., 2001) was used as a positive control (Fig 25b). As a non-specific negative control, a 287 bp *lacZ* fragment was used (Fig 25c). The negative control *lacZ* fragment is the largest of the all four fragments to exclude effects of unspecific DNA binding which are usually more pronounced for larger fragments.

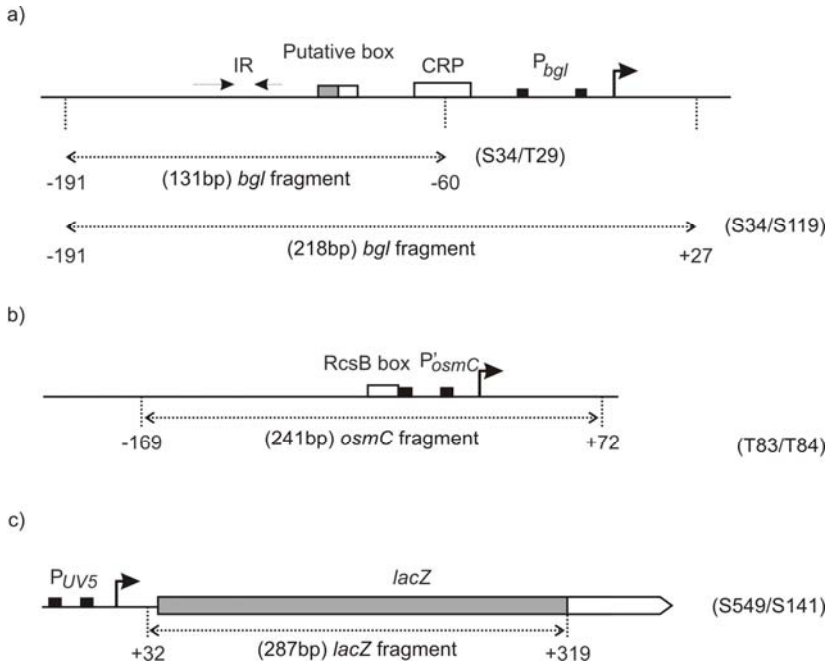


Fig 25. DNA fragments used for EMSA analysis. The fragments used for binding analyses are schematically shown. In all schemes, the -10 and -35 promoter regions are shown as small black boxes and the transcription start site is shown by an arrow. **a)** structure of the *bgl* regulatory region including the putative RcsB/BglJ box, shown as grey/white box. The white part represents the half matching RcsAB consensus sequence. The CRP binding sites is also indicated: The smaller 131bp *bg* frgment and larger *bgl* fragment are shown. The oligos used for amplification are mentioned; **b)** structure of the *osmC* regulatory region including the RcsB box. P'*osmC* represents the RcsB regulated *osmC* promoter1 (*osmCP1)***c)** structure of the *lac* promoter *lacZ* region with the DNA fragment used as negative control indicated.

At first, an EMSA was carried out by incubating the *bgl* and *lacZ* DNA fragments with increasing concentration of purified strep-tagged RcsB, $RcsB_{D56E}$ and $RcsB_{D56N}$ proteins. In the EMSA experiments the fragments were added at a final concentration of 15ng per assay (see materials and methods 17). The RcsB and the mutants did not shift *bgl* fragment (Fig 26). Even the positive control fragment *osmC* was not shifted by RcsB and its mutants.

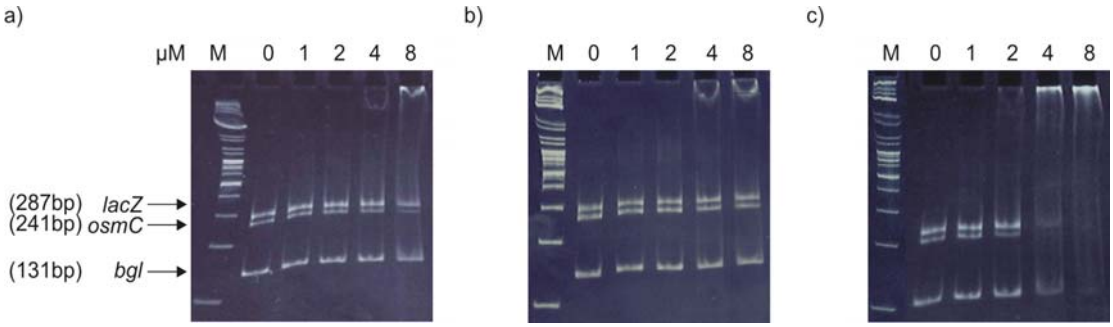


Fig 26. EMSA shift assay with purified RcsB and mutants. EMSA shift assay with purified RcsB (a), $RcsB_{D56E}$ (b) and $RcsB_{D56N}$ (c) proteins using *bgl, osmC* and *lacZ* DNA fragments. The RcsB and mutant proteins were added with the indicated final concentration to the fragment mixture and incubated for 20 minutes at 30ºC temperature. M is size standard (gene ruler, MBI fermentas). The protein-DNA complex is resolved on a 8% non-denaturing polyacrylamide gel and stained with ethidium bromide (0.5µg/ml) for 20 minutes. The DNA fragments (*bgl* 131bp), (*osmC* 241) and *lacZ* (147bp) are indicated by arrows.

An unsuccessful attempt to show the direct binding of RcsB to *osmC* by DNA shift assay was reported earlier (Davalos-Garcia et al., 2001). It was proposed that the affinity of binding is too low to obtain a stable complex during the Electrophoretic migration. We speculated that an additional cofactor might be required to establish a stable interaction of the protein to the target DNA.

The binding of H-NS to the *bgl* region was shown previously form our group (Dole et al., 2004) and we tried to analyze H-NS as a possible cofactor for the interaction of RcsB with it target genes. To check the effect of H-NS on the binding, I incubated the DNA fragments with increasing concentration of H-NS. As expected, the *osmC* fragments disappeared with increasing concentration of H-NS and at 1.2 µM H-NS it was completely shifted (Fig 27 lane d). H-NS also shifted the *bgl* fragment very effectively at the same concentration (Fig 27 lane d). Binding of H-NS in EMSA is usually detected by the disappearance of the unbound fragment and by the formation

of a shifted complex which appears as a smear due to oligomer complex formation of H-NS at the DNA

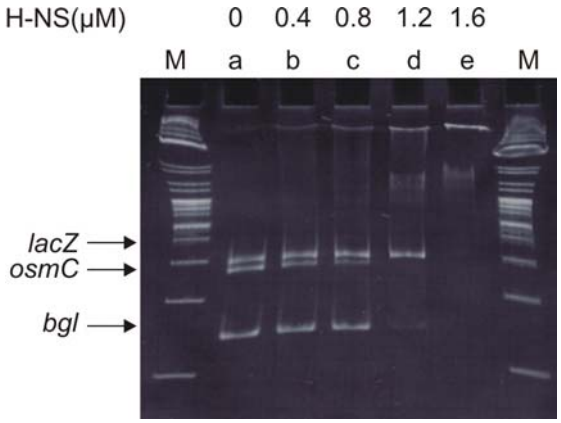


Fig 27. EMSA shift assay with purified H-NS protein using *bgl*, *osmC*, and *lacZ* DNA fragments. H-NS was added with the indicated final concentration to the fragment mixture and incubated for 20 minutes at 30ºC temperature. M is size standard (gene ruler, MBI fermentas). The protein-DNA complex is resolved on a 8% non-denaturing polyacrylaminde gel and stained with Ethidium bromide (0.5 μg/ml) for 20 minutes. The DNA fragments (*bgl* 131bp), *osmC* (241bp) and *lacZ* (287bp) are indicated by arrows.

In the next assay, we used increasing concentration of H-NS (0, 0.2, 0.4 and 0.8μM) and titrated against purified RcsB and mutant proteins. The RcsB protein showed a weaker shift in *osmC* fragment in the presence of H-NS at 0.4μM concentration (Fig 28.a (a-d). The 0.4μM H-NS concentration was not sufficient enough to shift the *osmC* fragment by alone (See Fig 27 b). The *osmC* shift was effective in the 0.8μM H-NS (Fig 28.a (f-h). Similar effects were seen with RcsBD56E protein (Fig 28.b (a-h). The RcsBD56N protein could able to shift *osmC* fragment only at 0.8μM concentration of H-NS (Fig 28.c (f-h). The lower concentrations of H-NS did not have any significant effect on shifting *omsC* or *bgl* fragment (Fig 28 a, c and e).

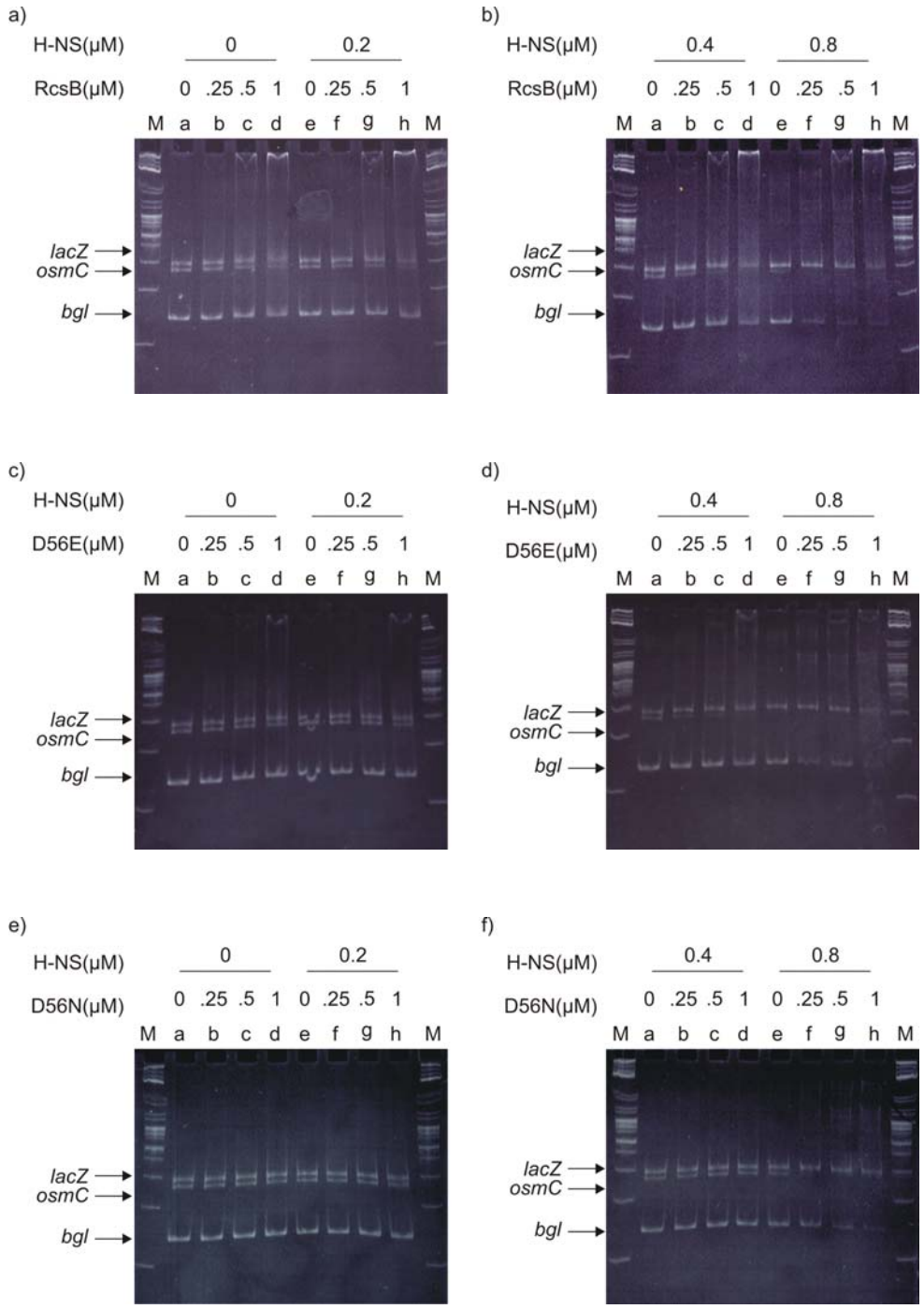


Fig 28. Effect of H-NS in RcsB and mutants binding. EMSA shifts in the presence of H-NS with RcsB (a,b), $RcsB_{D56E}$ (c,d) and $RcsB_{D56N}$ (e,f) is shown. H-NS and RcsB were added with the indicated final concentration to the fragment mixture and incubated for 20 minutes at 30ºC temperature. M is size standard (gene ruler, MBI fermentas). The protein-DNA complex is resolved on a 8% non-denaturing polyacrylaminde gel and stained with Ethidium bromide (0.5 μg/ml) for 20 minutes. The DNA fragments (*bgl* 131bp), *osmC* (241bp) and *lacZ* (287bp) are indicated by arrows

Finally, I performed EMSA assays using the same conditions in the presence and absence of partially purified BglJ-FLAG tagged protein. A constant amount of

partially purified BglJ protein (2µl) was used throughout the assay. The H-NS concentration was reduced to 0.6µM (since at 0.8µM concentration the protein-DNA complex sticks in the wells (Fig 28b, d and f). This suggests either the protein to DNA ratio is higher or the protein-DNA complex was too big for the gel. Here I used a 218bp *bgl* fragment which encompasses RcsB/BglJ putative site and core *bgl* promoter region (Fig 25a). The effect of the BglJ protein in shifting *bgl* fragment in the presence of H-NS (Fig 29.a (b-e) were inconclusive. Similar results were also observed with $RcsB_{D56E}$ and BglJ proteins in the presence of H-NS (Fig 29b (lane b - d). Since the BglJ protein purification was limiting, we cannot come to any conclusion about the effect of BglJ in the presence of RcsB and H-NS. Moreover this experiment was carried out only once.

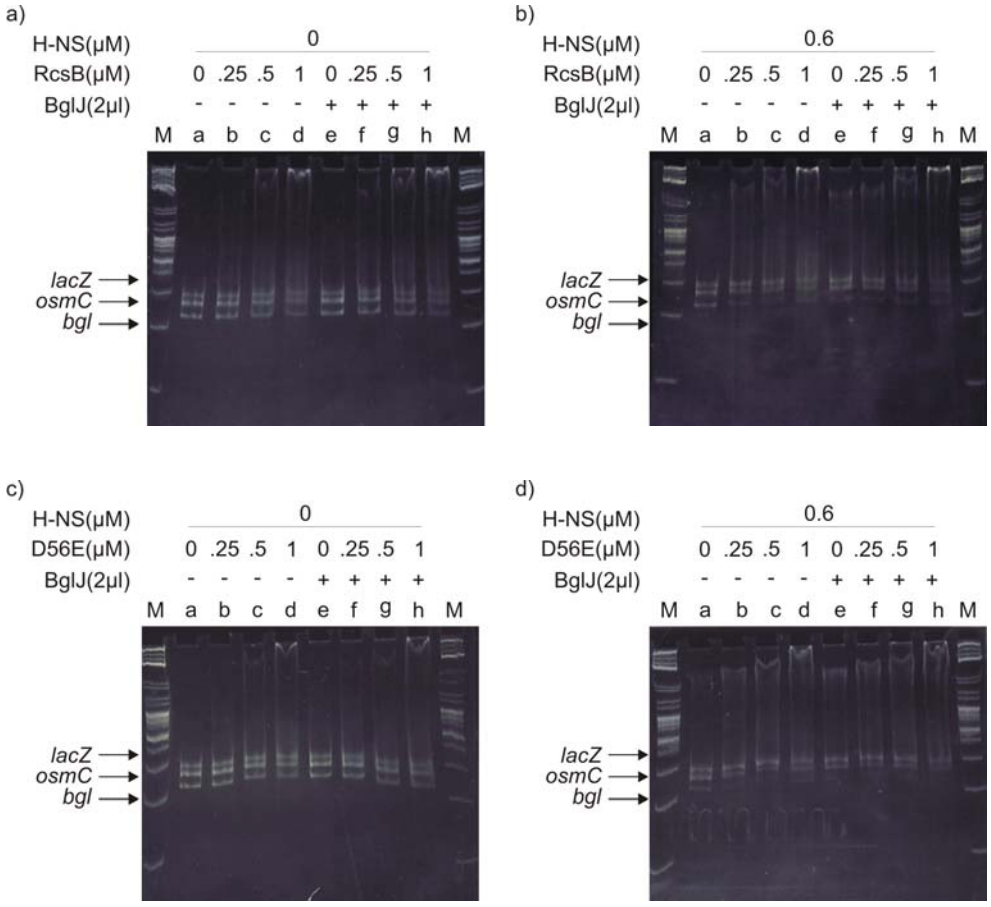


Fig 29. Effect of BglJ and H-NS in RcsB and mutants binding EMSA shifts in the presence of BglJ and H-NS with RcsB (a,b) and $RcsB_{D56E}$ (c,d) are shown. H-NS and RcsB were added with the indicated final concentration to the fragment mixture and incubated for 20 minutes at 30ºC temperature. M is size standard (gene ruler, MBI fermentas). The protein-DNA complex is resolved on a 8% non-denaturing polyacrylaminde gel and stained with Ethidium bromide (0.5 µg/ml) for 20 minutes. The DNA fragments (*bgl* 131bp), *osmC* (241bp) and *lacZ* (287bp) are indicated by arrows.

3. BglJ, a protease target in *E. coli*

The LuxR type transcription regulator BglJ, forms weak homodimer and strong heterodimer with RcsB. It has also been shown that the BglJ protein acts as antagonist (anti-silencer) of *bgl* operon silencing by H-NS, and that this is RcsB dependent (Paukner, 2007); unpublished data). The response regulator RcsB forms a heterodimer with the less stable auxiliary protein RcsA to activate transcription of capsule synthesis (*cps*) in *E. coli* and of other genes (Stout and Gottesman, 1990). Expression of the *rcsA* gene is also repressed by H-NS (Sledjeski and Gottesman, 1995). In addition, RcsA is rapidly degraded by Lon protease, and genetic data suggest that RcsA is stabilized by its interaction with RcsB (Gottesman and Stout, 1991; Torres-Cabassa and Gottesman, 1987; Dierksen et al., 1994; Stout et al., 1991). Later it was shown that RcsA in addition is a substrate of HslUV Protease (Kuo et al., 2004). Since BglJ is also forming a heterodimer with RcsB, I tested the possibility that BglJ is a target of proteases. This hypothesis is supported by the protein expression analyses, where BglJ protein expression was minimal and over expression of the protein leads to probable aggregation. To address the question a protein stability assay was performed using BglJ-FLAG protein in *E. coli* wild type and Lon protease mutants.

3.1 BglJ protein stability

To check the stability of BglJ protein, a low copy plasmid (pKERV10) coding for *bglJ* was used to transform *E. coli* wild type, a *lon* mutant, a *rcsB* mutant, and a *lon rcsB* double mutant. The cells were grown till OD_{600} reached 0.5 and then expression was induced with 1mM IPTG for 30 minutes at 37ºC. Aliquots were taken before induction (at (t = -30) and after 30 minutes of induction (at t = 0) before addition of chloromphenicol (200µg/ml) to stop protein synthesis. Then aliquots were taken 10 minutes, 30 minutes and 60 minutes after chloramphenicol addition. The protein samples were resolved on 15% SDS-PAGE and analyzed further with Western blot using mouse anti-FLAG/anti mouse alexaflour® 680 antibodies (Fig 31). Quantification of the Western blot suggests that the BglJ protein is less stable in the wild type cells as compared to the *lon* mutant strain. In the wild type 60 minutes after chloramphenicol addition (at t = 60) only 20% of the protein was detectable as

compared to t=0. In comparison in the *lon* mutant 60% of the protein remained at t = 60. This suggests that BglJ is a Lon protease target. The BglJ protein stability was rather similar in the wild type and in the *rcsB* mutant. Interestingly, BglJ was unstable in the *rcsB lon* double mutant (20%). These results indicate that the interaction of BglJ with RcsB has little effect on BglJ stability. The fact that BglJ is unstable in the *lon rcsB* double mutant indicates that another protease in addition to Lon degrades BglJ and that this degradation is inhibited by the interaction of BglJ with RcsB in the *lon* mutant. Recently, it has been shown that the ClpYQ (HslUV) protease acts as a secondary protease, which in addition to *lon,* targets RcsA (Kuo et al., 2004).

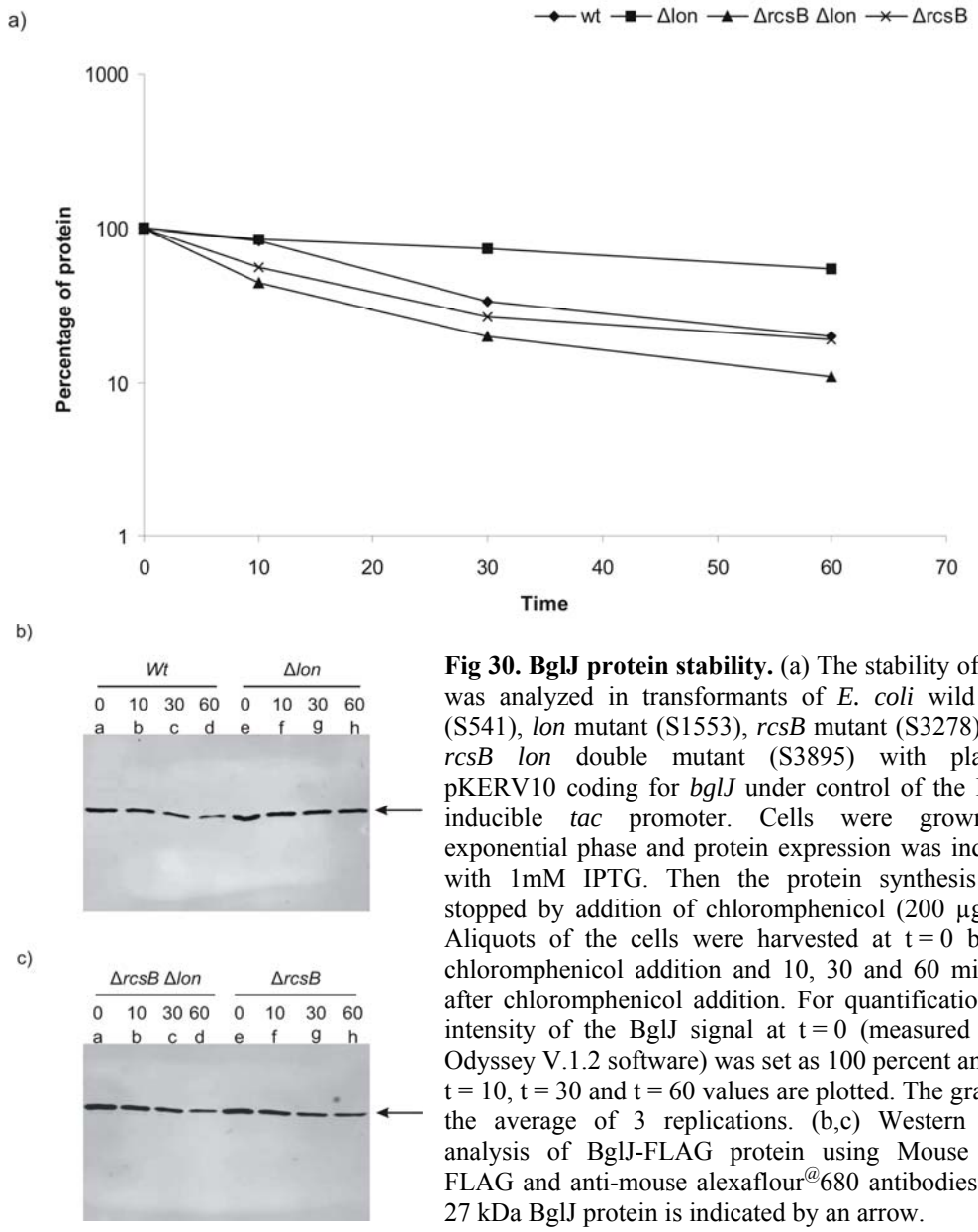


Fig 30. BglJ protein stability. (a) The stability of BglJ was analyzed in transformants of *E. coli* wild type (S541), *lon* mutant (S1553), *rcsB* mutant (S3278), and *rcsB lon* double mutant (S3895) with plasmid pKERV10 coding for *bglJ* under control of the IPTG inducible *tac* promoter. Cells were grown to exponential phase and protein expression was induced with 1mM IPTG. Then the protein synthesis was stopped by addition of chloromphenicol (200 µg/ml). Aliquots of the cells were harvested at t = 0 before chloromphenicol addition and 10, 30 and 60 minutes after chloromphenicol addition. For quantification the intensity of the BglJ signal at t = 0 (measured from Odyssey V.1.2 software) was set as 100 percent and the t = 10, t = 30 and t = 60 values are plotted. The graph is the average of 3 replications. (b,c) Western blots analysis of BglJ-FLAG protein using Mouse anti-FLAG and anti-mouse alexaflour@680 antibodies. The 27 kDa BglJ protein is indicated by an arrow.

4. Microarray

To identify genes in *Escherichia coli* regulated by the transcription factors BglJ and YjjQ, three different *E. coli* K12 strains were used, in which BglJ, YjjQ or as a control no protein was expressed. After induction of expression, RNA was isolated and used in a microarray analysis with Affymetrix Gene Chip *E.coli* Genome 2.0 array performed in Cologne Center for Genomics (CCG), Univeristy of Cologne, Germany.

E. coli strain S3922 (Δ*yjjP-yjjQ-bglJ*):: KD3cm) carrying deletions of *yjjQ* and *BglJ* was transformed with plasmids expressing BglJ (pKERV16), YjjQ (pKERV 17) genes under control of IPTG inducible *tac* promoter. The empty vector (pKES169) was used as a control. The cells were grown to mid stationary phase till OD_{600} reached 0.3 (~30 minutes) and induced with 1mM IPTG. The cells were grown till OD_{600} reached 0.5 (additional 30 minutes) and immediately harvested for RNA isolation using Qiagen RNAeasy kit. The RNA quality and concentration was checked with Nanodrop spectrophotometer (Nanaodrop ND-1000). For each strain 5 independent biological replicates of RNA was prepared for microarray analysis using Affymetrix GeneChip E.coli Genome 2.0 Array. The integrity of the RNA was analyzed on 6% Urea/acrylamide gel (Fig 31). The Microarray results are yet to be validated to confirm the results and avoid false positive results by real-time PCR.

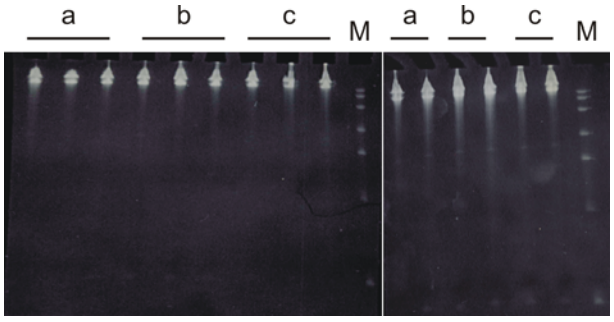


Fig 31. RNA preparation used in microarray analysis. RNA preparation from an E. coli strain (S3922 (MG1655 rph^+ Δ *(yjjP-yjjQ-bglJ)*:: KD3cm expressing BglJ (a), YjjQ (b) and control plasmid (c) was shown. A minimum 5 independent set of RNA was prepared for affymetrix Genechip E. coli Genome2.0 array. 1µg of purified RNA was separated in 6% Urea/acrylamide gel and stained with ethidium bromide (0.5µg/ml) for 10 minutes.

Though the microarray data are yet to be validated, the raw data from the array was analyzed based on the properties of the genes and only the significantly regulated

genes were shown in table1. As expected the bglJ gene was highly up regulated (2297 fold). Interestingly, the LysR-type transcriptional regulator LeuO, which antagonize H-NS repression was also highly up regulated (111 fold). Several inner and outer membrane proteins and structural proteins were positively regulated. The *rhs* elements form the Rhs super family with an unknown function was highly up regulated. Recently, *rhsA* had been shown to play a role in group 2 capsular polysaccharide biosynthesis (McNulty et al., 2006). Genes involved in iron transport were also regulated. Similarly, a significant down regulation in genes involved in acid resistance system is seen. The genes involved in stress response and oxidation/reduction system are also negatively regulated. Some non-coding small RNA are up and down regulated. The significant regulation of genes involved in various processes suggesting a wider role of BglJ protein other than activation of *bgl* operon.

Table1. Most significantly up or down regulated genes by BglJ protein

Gene	Gene products	Fold change
Transcriptional regulators		
bglJ	transcriptional regulator	+2297
leuO	regulator of leucine operon *leuabcd*	111
tdcA	regulator of *tdcabcdefg* operon	-12
gadW	regulator of glutamate acid resistance gene	-8
gadX	regulator of glutamate acid resistance gene	-5
caiF	regulator of *caitabcde* operon	-6
yhiF	response regaltor of decarboxylate transport system	-7
Inner and Outer membrane proteins		
yiaB	hypothetical inner membrane protein	+161
yiaA	hypothetical inner membrane protein	+75
yibG	hypothetical inner membrane protein	+60
yibJ	hypothetical inner membrane protein	+57
ycbS	outer membrane protein	+10
ybfC	outer membrane protein	+25
ompN	outer membrane protein	+17
Slp	outer membrane protein	-12
yigG	inner membrane protein	+43
yigF	inner membrane protein	+19
ybfB	inner membrane protein	+56
ygiZ	inner membrane protein	+48
yhiP	inner membrane protein	-11

Iron uptake		
fhuA	ferricchrome outer membrane transporter	+10
fhuF	ferricchrome reductase	+9
Fes	ferric eneterobactin esterase	+4
fepC	iron enterobactin atp binding protein	+3
fepB	iron enterobactin trasnporter binding protein	+3
Structural genes		
ycbQ	fimbrial like protein	+34
ycbR	pilin protein	+18
yadN	adhesion protein	+8
Rhs elements		
rhsB	rhs element core protein	+48
rhsA	capsular polysaccharide biosynthesis and metabolism	+37
rhsC	rhs element core protein	+31
Acid resistance system		
gadA	glutamate decarboxylase isoenzyme (gad)	-30
gadB	glutamate decarboxylase isoenzyme	-27
gadE	transcriptional activator of gad system	-17
gadC	glutamate gaba-antiporter	-13
hdeA	acid resistance protein chaperone	-21
hdeB	acid resistance protein chaperone	-14
hdeD	acid resistance protein chaperone	-9
Stress response (σ^s)		
osmY	osmotically inducible periplasmic protein	-4.5
osmE	osmotically inducible periplasmic protein	-2.5
osmF	osmotically inducible periplasmic protein	-2.5
Transporters		
yhiD	$mg2^+$ transporter	-13
yhiP	peptide transporter	-11
yhiU	multidrug resistance efflux transporter	-7
Oxidation/reduction system		
ydhY	ferridoxin like protein	-14
ydhV	predicted oxidoreductase protein	-11
hyaB	hydrogenase subunit	-13
hyaC	hydrogenase subunit	-11
hyaD	hydrogenase subunit	-6
		-5

Small regulatory RNA

micC	porin regulating RNA	+5
glmY	regulator of amino-sugar metabolism	+4
rhyB	regulator of iron homeostasis	+3
csrB	regulator of *csrA*(global pleiotropic regulator)	-4
gadY	activator of glutamate acid resistance genes	-3
dsrA	activator of *rpoS* regulator of *hns*	-3

Table1. The affymetrix microarray of BglJ protein. The plasmid expressing BglJ (pKERV16) under the IPTG inducible *tac* promoter was grown till 0.3 OD600 and induced with 1mM IPTG for additional 30 minutes. The RNA was isolated and the gene expression analysis was carried out using Affymetrix Gene chip E. coli Genome 2.0 array. The empty vector (pKES169) was used as a control. For each strain 5 independent biological replicates of RNA was prepared. The genes which are significantly regulated by BglJ protein (BglJ vs control genes) are shown in table1.

IV. Discussion

The nucleoid associated protein H-NS acts as a global transcriptional repressor controlling approximately 5% of all genes in *E. coli* and other enterobacteriaceae (see introduction). Repression by H-NS can be relieved by binding of specific transcription factors and by alteration in the DNA structure (Stoebel et al., 2008).One model system for repression by H-NS is the *E. coli bgl* operon, which is effectively repressed by H-NS (Dole et al., 2004; Madhusudan et al., 2005; Nagarajavel et al., 2007). This repression can be relieved by the LuxR-type transcription factor BglJ. De-repression by BglJ requires the response regulator and LuxR-type transcription factor RcsB. In addition, it was shown by a bacterial two-hybrid system that BglJ and RcsB form heterodimers (unpublished lab data). Therefore, it seemed plausible that BglJ-RcsB heterodimers bind to the regulatory region of the *bgl* operon and antagonize repression by H-NS. In this Thesis heterodimer formation of BglJ with RcsB was confirmed by Co-immuno precipitation analysis. Additional, protein stability analysis suggests that BglJ is a target of Lon and another protease. Furthermore, binding of the BglJ-RcsB dimer to the *bgl* regulatory region is supported by the analysis of linker insertion mutants within and close to a putative BglJ-RcsB binding site. In addition, binding was tested *in vitro* by electrophoretic mobility shift assays using purified RcsB and a protein fraction enriched for BglJ, and by other approaches. The DNA shift assays which included a control fragment (*osmC*) known to be bound by RcsB homodimers suggest that binding of RcsB requires a minimum amount of H-NS, indicating that H-NS might act as a cofactor. Binding of RcsB-BglJ to the *bgl* fragment was not conclusive most likely because purification of BglJ was of limited success. Taken together, the results of this experiments support a model that H-NS act as a cofactor increasing the affinity of RcsB/RcsB homodimers (for RcsB regulated loci) and also for RcsB/BglJ heterodimers (for *bgl*) which leads to de-repression of the *bgl* operon.

1. RcsB interaction with BglJ and YjjQ

The co-immunoprecipitation demonstrated efficient interaction of BglJ with the wild type RcsB. Similar results were obtained from the interaction analysis of YjjQ with wild type RcsB protein. Interaction of YjjQ with RcsB was 3.2 fold less efficient than

interaction of BglJ with RcsB. This result is also well supported form the data obtained from the bacterial two-hybrid system. YjjQ was included in these analyses as it is encoded in an operon with BglJ. In addition co-immunoprecipitation of BglJ and YjjQ were performed, with RcsB mutants carrying exchanged in the conserved Aspartate phosphorylation site. For BglJ these results are contradictory to results obtained with an *in vivo* bacterial two-hybrid system. In the two-hybrid system, interaction of RcsB mutant proteins with BglJ showed no significant difference than the wild type. On the other hand, YjjQ showed a stronger interaction with $RcsB_{D56E}$ than the wild type. The $RcsB_{D56N}$ mutant showed the weakest interaction with YjjQ. A possible explanation for the difference in the interaction levels of RcsB and its mutants with BglJ and YjjQ could be attributed to the poor expression of the BglJ and YjjQ, respectively (fig 6). Due to the poor protein levels, it is possible that not enough proteins were available to form a complex with RcsB. In addition, RcsB forms a strong homodimer. If the $RcsB_{D56E}$ forms stronger homodimer, one can imagine that most of the proteins would be used in homodimer formation and less protein would be available for heterodimerization with BglJ or YjjQ.

As far as our present knowledge the phosphorylation site RcsB proteins was never tested biochemically. RcsB has a conserved aspartate residue at position 56, which is assumed to be the phosphorylation site by analogy to other response two-component system regulators like CheY (chemotaxis protein), NtrC (nitrogen regulatory protein) and VirG (conjugation protein in *Agrobacterium tumefaciens*) in which the corresponding aspartate residue was shown to be the site of phosphorylation (Bourret et al., 1990; Keener and Kustu, 1988; Klose et al., 1993; Jin et al., 1990). In addition, genetic data support that D56 is the phosphorylation site; the change of aspartate to glutamate (D56E) leads to constitutive activation of *cps* gene, and aspartate to asparagine (D56N) results leads to failure to activate the *cps* gene (Stout, 1994). An in depth analysis of phosphorylation of RcsB should be done through mass spectrometry analysis. The binding affinity of RcsB and the mutant proteins to the interaction partners could be analyzed by techniques like Surface Plasmon Resonance (SPR) to get an idea about the role of phosphorylation status in the interaction.

2. De-repression of the *bgl* operon by BglJ-RcsB might require H-NS as a cofactor

The identification of a 7 bp sequence motif in the upstream *bgl* regulatory region matching half of the RcsAB consensus sequence suggested a RcsB/BglJ binding site in the *bgl* regulatory region (Paukner, 2007). Here, linker insertion mutants were used to analyze whether the integrity of this putative binding site is required for activation by BglJ/RcsB. In the *E. coli* strain S541 (Δ*bgl* ΔlacZ) the wild type *bgl* promoter is repressed by H-NS, while its activity increased approximately 88 fold when BglJ was expressed which shows de-repression by BglJ (Fig 12b). This de-repression was abolished in the *rcsB* deletion, which shows that the de-repression of BglJ is RcsB dependent. Linker insertion in the putative binding site and between the putative binding site and promoter region abolished the activation by BglJ/RcsB. In addition, a linker insertion upstream of the binding site also interfered with activation by BglJ-RcsB. These results supported our hypothesis that the BglJ/RcsB heterodimer binds to the *bgl* regulatory region to antagonize H-NS repression.

To support the hypothesis that BglJ/RcsB heterodimers bind to putative site in *bgl*, I tried many biochemical approaches including Chromatin immunoprecipitation (CHIP), electrophoretic mobility shift assay (EMSA) using cell lysates and a modified protocol of co-immunoprecipitation which includes addition of the *bgl* regulatory fragment in the lysates followed by a precipitation with HA specific antibodies. All these experiments yielded mostly non-specific interactions and the results were inconclusive. Another genetic approach, based on a bacterial one-hybrid system for determining the DNA-binding specificity of transcription factors was also not convincing. The controls showed a 3 fold increase in activation (Fig 14b). The bacterial one-hybrid system expressing RcsB/BglJ putative binding site plasmids (direct and inverse orientation), showed a 2 fold increase in the activity in plasmids carrying putative binding site in inverse orientation (pKERV53 to pKERV56) (Fig 17b) but this effect was not observed in same strain expressing empty vector control (Fig 18). We could not understand the difference. Moreover the high basal level expression makes it difficult to analyze the induction effects (See Results 2.2).

In the parallel approach, electrophoretic mobility shift assay (EMSA) with purified RcsB and RcsB mutant proteins shifted neither the *bgl* fragment nor the *osmC*

fragments (with the latter being used as a control as it is known to be bound by RcsB homodimers). At this point we were not sure weather this is due to loss of protein function or activity or due to technical error. But it should be considered that in the literature *in vitro* binding of RcsB to its target genes like *osmC* and *fts* (cell division gene) was also problematic (see for example (Davalos-Garcia et al., 2001)). Moreover, RcsB binding to its target gene by electrophoretic DNA shift assay has not been demonstrated so far. Binding site analysis were successful only with DNAse I footprinting for RcsB target genes (Sturny et al., 2003). Interestingly, DNA shift assays of RcsB with its auxiliary protein RcsA has been shown for several RcsB/RcsA target genes (Kelm et al., 1997; Francez-Charlot et al., 2003; Wehland et al., 1999; Wehland and Bernhard, 2000). It is reported that LuxR, a transcriptional activator of *Vibrio fischeri* which shares the same family of response regulators like RcsB is unable to bind to its target genes alone (Stevens et al., 1994). It is not clear why binding cannot be established by DNA shift assays.

It could be possible that the affinity of binding is too low to obtain a stable complex during the electrophoretic migration. Another possibility is that an additional cofactor is required to establish a stable interaction of the RcsB/BglJ proteins to the target DNA. Both *bgl* and *osmC* are repressed by H-NS (Schnetz, 1995; Dole et al., 2004; Gutierrez and Devedjian, 1991)., and, as expected, *bgl* and *osmC* fragments were shifted with purified H-NS protein (Fig 27). Therefore, I tested whether H-NS acts as a co-factor for binding of RcsB to *osmC* and RcsB-BglJ to *bgl*. Upon incubation of the DNA fragments with purified H-NS and RcsB proteins the *osmC* fragment was effectively shifted. The concentrations of H-NS and RcsB proteins used were insufficient to shift *osmC* and *bgl* fragments alone (fig 28). This indicated that H-NS acts as a co-factor of RcsB binding, which could be validated by shift assays with fragments carrying mutations of the RcsB binding site. To my knowledge this is the first time that the direct binding of RcsB to the *osmC* promoter region is shown. In addition, no significant difference in the shift of the *osmC* fragment by RcsB was observed for the wild type RcsB or its D56 mutants (fig 28).

Based on these results, one may assume that a limited amount of H-NS is required for enhancing the affinity of RcsB or by increasing the stability of interaction by forming H-NS-RcsB-DNA complex. The transcriptional repressor H-NS has been shown to positively activate *tra* gens which encode products required for conjugative transfer

like F-like plasmids. H-NS has been shown to bind directly along with another global regulator Lrp (Leucine response regulatory protein) and activates the transcription (Starcic-Erjavec et al., 2003). Though the specificity of binding was not high as Lrp and the mechanism by which the H-NS and Lrp enhances the *tra* promoter activity remains elusive. A transcriptional regulator *slyA*¸ has been shown to relieve H-NS mediated repression of a heamolysin gene *hlyE* in *E. coli* (Westermark et al., 2000; Lithgow et al., 2007). The shifts using purified H-NS and RcsB proteins along with constant amount of partially purified BglJ protein were not conclusive. This could be due to the problem with the homogeneity of the partially purified BglJ protein The BglJ protein might not be sufficient enough to contribute to the DNA-protein complex.

3. Lon protease targets BglJ

The BglJ protein stability experiment (Fig 30) showed that BglJ is targeted by Lon protease. It has been shown that many LuxR-type transcriptional regulators like LuxR regulator in *V. fischeri*, PpuR transcription factor in *P. aeruginosa,* TraR and RcsA in *E.coli* are degraded by Lon protease and many of them involved in pathogenicity (Manukhov et al., 2006; Bertani and Venturi, 2004; Stout et al., 1991; Zhu and Winans, 2001). The stability of BglJ in the wild type and *rcsB* mutant is similarly low, indicating that the presence of RcsB has no effect on the stability of BglJ. This result contradicts a hypothesis that interaction of BglJ with RcsB increases the stability of the BglJ protein, since heterodimer formation might prevent the protein form degradation by Lon protease. Presently, it is not know weather phosphorylated RcsB (which can be tested with the mutant $RcsB_{D56E}$) could have had an increased effect on the stability of the BglJ protein. In addition, instability of BglJ in the *rcsB lon* double mutant suggests that another protease is involved in the degradation of BglJ, and that in this case RcsB has an affect on BglJ stability. In bacteria, regulated degradation is carried out mainly by two major ATP-dependent proteases, Lon and Clp (Baker and Sauer, 2006; Striebel et al., 2009). It has been recently shown that the additional ClpYQ (HslUV) protease acts as a secondary protease in addition to Lon in the degradation of RcsA (Kuo et al., 2004). Therefore, ClpYQ could be the secondary protease which degrades BglJ, which has to be experimentally analyzed.

One interesting aspect that remains unclear is how BglJ activity and its proteolysis are controlled. BglJ was initially identified as an activator of the *bgl* operon. The results of the microarray and additional data obtained in the lab revealed that BglJ is a global RcsB dependent regulator. This is also further supported by a fact that the over expression of BglJ results in a growth defects (unpublished lab data). The microarray showed significant regulation of genes involved in acid resistance and stress response along with structural proteins, inner and outer membrane proteins. The significant regulation of the potential candidates suggests a widespread role of BglJ protein.

4. Model

Based on the results, one can propose a model for anti-silencing of the H-NS repressed *bgl* operon by transcriptional factors RcsB and BglJ. In this model, it is assumed that a limited amount of H-NS is required as a cofactor for RcsB/BglJ binding to its binding site in *bgl*. The presence of H-NS might enhance the affinity of binding or increase the stability of the interaction by binding to the RcsB/BglJ heterodimer. It is also possible that binding of H-NS to the DNA causes a conformational change in the RcsB/BglJ site, which could facilitate binding of the RcsB/BglJ heterodimer. Binding of RcsB/BglJ heterodimer may prevent the H-NS nucleoprotein formation and DNA-HNS-DNA bridging, and thus prevent inhibition of RNA polymerase by H-NS. In addition, direct interaction of BglJ-RcsB with RNA polymerase may by important for activation, as linker insertions in between the putative BglJ/RcsB binding site and the promoter prevent activation.

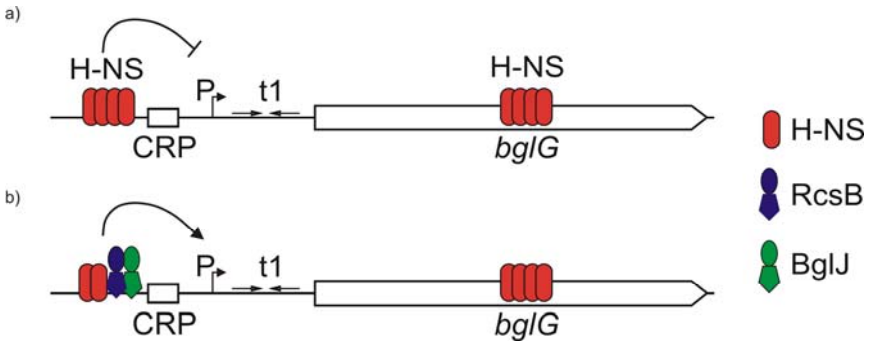


Fig32. Model of anti-silencing of H-NS repressed *bgl* operon by transcriptional factors RcsB and BglJ. a) The H-NS mediated repression of *bgl* operon shows the binding of H-NS (red) to the Upstream and downstream regulatory element. (b) A minimum amount of H-NS is bound with RcsB/BglJ prevents the formation of nucleoprotein complex and DNA-H-NS-DNA bridging.

V. Materials and methods

1. Media and agar plates

LB (1000ml)

Bacto Trypton	10g
Yeast Extract	5g
NaCl	5g

For LB plates add 15g Bacto Agar (1.5%)

SOB (1000ml)

Bacto Tryptone	20g
Bacto Yeast Extract	5g
NaCl	0.5g
2M KCl	1.25ml

Adjust the pH to 7.0 with NaOH
After autoclaving just before use add 10ml 1M $MgCl_2$

SOC (1000ml)
Add 19.8ml 20% Glucose to 1000ml SOB

20 x M9

Na_2HPO_4 x 2 H_2O	140 g
KH_2PO_4	60 g
NH_4Cl	20 g

Make up the volume to 1000ml with sterile H_2O

M9 Medium (prepare from sterile solutions)

20 x M9	50 ml
0.1 M $CaCl_2$	1 ml
1 M $MgSO_4$	1 ml
1 mM $FeCl_3$	0.5 ml

Add carbon source 1% final concentration:

20 % Glucose	50 ml
or 80 % Glycerol	12.5 ml

If required:

1 mg/ml Vitamin B1	1 ml
4 mg/ml amino acids	5 ml
10% casamino acids	66 ml

Make up the volume to 1000ml with H_2O

M9-plates
For M9 plates add 15g Bacto Agar (1.5%) with 900ml H_2O
Add, sterile

20 x M9	50 ml
0.1 M $CaCl_2$	1 ml
1 M $MgSO_4$	1 ml
1 mM $FeCl_3$	0.5 ml

Carbon source: 1 % final concentration

Add, if required:

1 mg/ml Vitamin B1	1 ml
4 mg/ml amino acids	5 ml
10% casamino acids	66 ml

Bromthymol blue plates (BTB-plates)

Bacto Agar	15g
Yeast-Extract	1g
Trypton	1g
NaCl	5g
H_2O	900 ml

Autoclave at 121ºC for 20minutes

Add sterile:

1 M MgSO4	1 ml
0,1 M CaCl2	1 ml
Vitamin B1	1 ml

(stock solution 1mg/ml, filter sterilize)

FeCl3 1mM	0,5 ml
10% (w/v) Casaminoacids	20 ml
Sugar	50 ml

(e.g. 10 % Salicin, 20% Lactose, etc.)

BTB stock solution	10 ml

(2% bromthymol blue in 50% EtOH, 0,1N NaOH)

Antibiotics if required.
The medium should be turquoise,
if medium is green add NaOH, if it is blue add HCl

2. Antibiotics, sugars, Amino acids

Antibiotics

Name	Stock solution	Final conc.
ampicillin	50mg/ml in 50 % EtOH	50 µg/ml
chloramphenicol	30 mg/ml in Ethanol	15 µg/ml
kanamycin	10 mg/ml in water	25 µg/ml
rifampicin	100mg/ml in Methanol	100µg/ml
spectinomycin	50 mg/ml in 30% EtOH	50µg/ml
tetracyclin	5mg/ml in 70 % Ethanol	12 µg/ml

Sugars

Name	Stock solution	Final conc.
Glucose	20%	1%
Glycerol	80%	1%
Salicin	10%	0.5%

Casamino acids

Final conc. 10% in H_2O

Dissolve by heating and filter through Schleicher & Schuell folded filters, then autoclave.

3. Buffers TEN Buffer

20mM Tris.Hcl (pH7.5)
1mM EDTA
50mM NaCl

1X Laemmli Buffer

62.5mM Tris.Hcl (pH6.8)
2% SDS
10% Glycerol
0.05% Bromophenol blue
5% β-mercaptoethanol

Z-Buffer (β-galactosidase assay)

100mM Na-phosphate pH 7.0
10mM KCl
1mM MgSO4
100µg/ml chloramphenicol)

EMSA Binding Buffer

20mM Tris-HCl pH 7.5
100mM KCl
2mM DTT
10% glycerol

EMSA loading dye (6X)
10 mM Tris-HCl pH 7.6
0.03% bromophenol blue
0.03% xylene cyanol FF
60% glycerol
60 mM EDTA)

10x TBE (1Liter)
20 ml of 0.5 M EDTA pH 8.0
27.5 g boric acid
54 g Tris base
800 mL H_2O

Buffer A (FPLC)
50mM Tris-HCl pH 8.0
100mM KCl
1mM EDTA

Buffer B (FPLC)
50mM Tris-HCl pH 8.0
100mM KCl
1mM EDTA
2.5mM D-Desthiobiotin

Buffer R (FPLC)
50mM Tris-HCl pH 8.0
100mM KCl
1mM EDTA
1mM HABA

Dialysis Buffer
20mM Tris-HCl pH7.5
100mM KCl
2mM DTT
10% Glycerol
1mM PMSF

4. Standard microbiology techniques

Standard Molecular Biology applications like restriction enzyme digestions, ligations and other enzymatic reactions, PCR amplification, plasmid purification, auto-radiography were performed as described in Sambrook and Russell, 2001 or current protocols (Ausubel FM, 2005) or according to the manufacturer's instructions.

5. Bacterial strains

The bacterial strains used in this study and their description are listed in table2

Table 2: *E. coli* K12 strains used in this study		
Name	**Relevant genotype or structure[a]**	**Source**
CSH50	=S49 (bgl° Δ(lac-pro) ara thi	(Miller, 1972)
S103	DH5α F- araD139 Δ(argF-lac)U169 deoC1 flb5301 relA1 rpsL150 ptsF25 rbsR	Lab collection
S524	CSH50 ΔlacZ-Y217 $(gpt\text{-}pro)^+$	(Dole et al., 2002)
S541	CSH50 (=S539) Δbgl-AC11 ΔlacZ-Y217	(Dole et al., 2004)
S1553	=S541 sulA3 Δlon $proC^+$	Lab collection
S2176	=S524 yjjQ/bglJ-Y6::mTn10cm	Lab collection
S2822	=S524 (S2817 yjjQ/bglJ-Y6::mTn10cm)	Lab collection
S2828	=S524 (S2822 rcsB::mTn10tet-2828)	Lab collection
S3278	=S541 ΔrcsB::SpecR	Lab collection
S3377	=S541 ΔrcsB::SpecR Δ□yjjP-bglJ)::KD3cmR	S783/S676, pKD3[c]
S3895	= S541 sulA3 Δlon $proC^+$ ΔrcsB::SpecR	S1553 x T4TG7[b]
S3922	=(MG1655 rph^+ Δ(yjjP-yjjQ-bglJ)::KD3-cm)	S3839/pKD46[c]
S4160	=S541 ΔrcsB::SpecR Δ(yjjP-bglJ)::KD3frt	S3377/pCP20[c]
S4191	= S541 attB::(SpecR lacIq rrnB-T1 OL2 Plac lacZ)	X pKERV45

a: The relevant genotype of the strains (which are all CSH50 derivatives) refers to the *bgl, lac.* All the strains were checked with PCR /restriction digestion and sequencing. The confirmed strains were stored (1.5ml of overnight culture mixed with 50µl DMSO (Dimethyl sulfoxide) at -80ºC. Detailed description of all the strains used was documented in lab records.
b: Construction of strains by transduction using T4GT7 is explained in materials and methods (V.6) and integration of plasmids into the *attB* site of chromosome was done as described (Diederich et al., 1992) (see materials and methods).
c: The chromosomal deletion of rcsB, *bglJ* allele was constructed according to (Datsenko and Wanner, 2000) and is explained in detail in material and methods (V.6). $\Delta yjjP\text{-}yjjQ\text{-}YjjQ::cam^R$ refers to the replacement of the chromosomal *yjjPQJ* gene by a chloromphenicol resistance gene cassette, which was amplified from plasmid pKD3.

6. Plasmids

Large scale preparations of plasmid DNAs were performed using the plasmid maxiprep/midiprep kit (Promega) according to manufacturer's instructions. A list of plasmids used in the study, with brief descriptions is given in the Table 2. Details of the plasmid constructions are documented in the lab records and sequences are compiled in Vector NTI (Invitrogen).

The plasmids used in the study were derivatives of pMB1, pSC101 or – derivatives (pMB1). The origin of each of the plasmids is also listed. The pMB1-derivatives carry the p15A origin of replication and the λ phage attachment site *attP*, to allow λ integrase mediated recombination insertion into the *attB* site of the *E.coli* chromosome (Diederich et al., 1992). The pSC101 derivatives used in the study carry the chloramphenicol resistance gene (*cat*), the *repA* gene and a *lacI*^{q} gene-*lacUV5* or *tac* promoter cassette, followed by a multiple cloning site. The *tac* promoter is flanked by two operators for efficient repression by the *lac* repressor (LacI). The plasmids pKK177-3 has a 322 based origin of replication (pMB1), ampicillin resistance gene (*bla*) and two strong Rho independent transcriptional terminators *rrnB*-T1 and T2 (Brosius and Holy, 1984).

The plasmids used in this study and their description are listed in table3

Table 3: Plasmids used in this study

Plasmid	Relevant structure/description/resistance[a]	Source
pCP20	FLP recombinae, temperature sensitive, amp	Datsenko KA, 2000
pKD3	Template plasmid for gene deletion, cm	Datsenko KA, 2000
pKD4	Template plasmid for gene deletion, kan	Datsenko KA, 2000
pKD46	λ red recombinase, temperature sensitive, amp	Datsenko KA, 2000
pLDR8	λ repressor, cI-857; int under control of λ P_R, pSC101 rep-ts kan	Diederich L 1992
pFDX733	*Wt bgl* operon kan	Schnetz K (1987)
pFDY127	lacI^{q} Ptac bglG, pBR amp	Lab Collection
pFDY167	bglP t1 bglG lacZ, p15A kan	Caramel.A (1998)
pKETS01	lacI^{q} Ptac bglJ, p15A kan	Lab collection
pKEAP22	lacI^{q} Ptac, pBR amp	Lab Collection
pKEAP38	lacI^{q} Ptac rcsB-HA, pBR amp	Lab collection
pKEAP43	lacI^{q} Ptac $rcsB_{D56E}$-HA, pBR amp	Lab collection
pKEAP44	lacI^{q} Ptac $rcsB_{D56N}$-HA in pBR amp	Lab collection
pKES169	lacI^{q} Ptac SD10 C-term-HA, p15A kan	Lab collection
pKES171	lacI^{q} Ptac SD10 C-term-HA, pBR amp	Lab collection
pKES179	lacI^{q} Ptac SD10 yjjQ-HA, p15A kan	This study
pKES181	lacI^{q} Ptac SD10 yjjQ-HA, pBR amp	This study
pKES182	lacI^{q} Ptac SD10 FLAG, p15A kan	This study
pKES183	lacI^{q} Ptac SD10 Myc p15A kan	This study
pKES184	lacI^{q} Ptac SD10 C-term-FLAG, pBR amp	This study
pKES185	lacI^{q} Ptac SD10 C-term-Myc, pBR amp	This study
pKETS01	lacI^{q} Ptac BglJ, p15A kan	Lab collection

pFMAC20	bglP^{o} t1 bglG lacZ, p15A kanR (SalI site at 249 bp upstream to CAP and single bp exchange (EcoRI) in URE	Caramel A, 1998
2-hybrid	lacUV5 α-σ 70 ^{D581G} , amp (positive control)	Hochschild A, 1999
2-hybrid	lacUV5 α , amp (negative control)	Hochschild A, 1999
2-hybrid	lacUV5 λcI- $\beta^{831-1057}$, p15A cm (negative control)	Hochschild A, 1999
2-hybrid	lacUV5 λcI, p15A cm (negative control)	Hochschild A, 1999
pKERV02	lacI^{q} Ptac SD10 yjjQ-FLAG, pBR amp	This study
pKERV04	lacI^{q} Ptac SD10 yjjQ-Myc, pBR amp	This study
pKERV06	lacI^{q} Ptac SD10 yjjQ-FLAG, p15A kan	This study
pKERV08	lacI^{q} Ptac SD10 yjjQ-Myc, p15A kan	This study
pKERV09	lacI^{q} Ptac SD10 bglJ-HA, p15A kan	This study
pKERV10	lacI^{q} Ptac SD10 bglJ-FLAG, p15A kan	This study
pKERV12	lacI^{q} Ptac SD10 bglJ-HA, pBr amp	This study
pKERV13	lacI^{q} Ptac SD10 bglJ-Myc, p15A kan	This study
pKERV14	lacI^{q} Ptac SD10 bglJ-FLAG, pBR amp	This study
pKERV15	lacI^{q} Ptac SD10 bglJ-Myc, pBR amp	This study
pKERV16	lacI^{q} Ptac bglJ, pMB1 kan	This work
pKERV17	lacI^{q} Ptac yjjQ, pMB1 kan	This work
pKERV18	PlacUV5 α-rcsB, pKK amp	This work
pKERV31	lacI^{q} Ptac strep-rcsB, pKK amp	This work
pKERV32	lacI^{q} Ptac strep-rcsB$_{D56E}$, pKK amp	This work
pKERV33	lacI^{q} Ptac strep-bglJ, pKK amp	This work
pKERV34	lacI^{q} Ptac strep-rcsB, p15A kan	This work
pKERV35	lacI^{q} Ptac strep-rcsB$_{D56E}$, p15A kan	This work
pKERV36	lacI^{q} Ptac strep-bglJ, p15A kan	This work
pKERV41	lacI^{q} Ptac strep-rcsB$_{D56N}$, pKK amp	This work
pKERV42	lacI^{q} Ptac strep-yjjQ, pKK amp	This work
pKERV43	lacI^{q} Ptac strep-rcsB$_{D56N}$, p15A kan	This work
pKERV44	lacI^{q} Ptac strep-yjjQ, p15A kan	This work
pKERV45	lacI^{q} rrnBT1 OL2 Plac lacZ, pAYC kan,cm	This work
pKERV46	lacI^{q} rrnBT1 OL2 Plac lacZ, pSC101 cm	This work
pKERV47	lacI^{q} rrnBT1 BglJ-RcsB box (-108 to -87) Plac lacZ, pSC101cm	This work
pKERV48	lacI^{q} rrnBT1 BglJ-RcsB box (-108 to -85) Plac lacZ, pSC101cm	This work
pKERV49	lacI^{q} rrnBT1 BglJ-RcsB box (-108 to -83) Plac lacZ, pSC101cm	This work
pKERV50	lacI^{q} rrnBT1 BglJ-RcsB box (-108 to -81) Plac lacZ, pSC101cm	This work
pKERV51	lacI^{q} rrnBT1 BglJ-RcsB box (-108 to -79) Plac lacZ, pSC101cm	This work
pKERV52	lacI^{q} rrnBT1 BglJ-RcsB box (-108 to -77) Plac lacZ, pSC101cm	This work
pKERV53	lacI^{q} rrnBT1 BglJ-RcsB box (-108 to -87) Plac lacZ, pSC101cm	This work
pKERV54	lacI^{q} rrnBT1 BglJ-RcsB box (-110 to -87) Plac lacZ, pSC101cm	This work

pKERV55	$lacI^q$ rrnBT1 BglJ-RcsB box (-112 to -87) Plac lacZ, pSC101cm	This work
pKERV56	$lacI^q$ rrnBT1 BglJ-RcsB box (-114 to -87) Plac lacZ, pSC101cm	This work
pKERV57	$lacI^q$ rrnBT1 BglJ-RcsB box (-116 to -87) Plac lacZ, pSC101cm	This work
pKERV58	$lacI^q$ rrnBT1 BglJ-RcsB box (-118 to -87) Plac lacZ, pSC101cm	This work
pKERV59	$lacI^q$ rrnBT1 BglJ-RcsB box (-120 to -87) Plac lacZ, pSC101cm	This work

All the plasmids mentioned here were cloned by digestion of the vectors and the fragments with corresponding restriction enzymes and gel purified using Qiagen gel purification kit. The ligated fragments were transformed into *E. coli* DH5α (S103) strain and the plasmids were confirmed by PCR amplification and restriction digestion followed by sequencing analysis. The correct plasmids were prepared in large scale using maxiprep/midiprep kit (Promega) according to manufacturer's instructions. The plasmids were stored at 4ºC.
a: plasmids which carry a pMB1 (p15A) origin of replication and kanamycin, spectinomycin resistance markers also harbor the *attP* site for integration into the chromosome according to (Diederich et al., 1992). Plasmids carrying origin of replication carry an ampicillin resistance marker. Detailed description of the plasmid construction is documented in lab records and the sequences are compiled in the lab Vector NTI (Invitrogen) database.

7. DNA sequencing

DNA sequencing was done with the Big dye terminator cycle sequencing kit (version 1.1, ABI prism) according to manufactures instruction and using automated DNA sequencer (The Cologne Center for Genomics, (CCG) Institute for Genetics, University of Köln). For sequencing the reaction was carried out in a total volume of 10µl with 1µl of big dye sequencing mix. Nucleotide sequence alignments were performed using the Vector NTI program (Invitrogen).

8. Deletion of genes according to Datsenko and Wanner

Deletion of genes was done according to Datsenko and Wanner (2000). This system is based on the λ Red based recombination between linear DNA fragment and the chromosomal gene. The basic strategy is to replace the chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR and by using primers with 30 to 50 nt homology extensions of the gene to be deleted. Briefly, the cells were transformed with temperature sensitive plasmid (pKD46) which has λ red system under the control of inducible arabinose promoter. The PCR product for deletion of a target gene was generated using primers carrying homology to the target chromosomal region and to antibiotic resistance cassettes of plasmids pKD3 and pKD4. This PCR generates a fragment carrying the chloramphenicol or kanamycin

resistance genes, flanked by a short homology to upstream and downstream sequences of the target gene. In addition, the resistance genes are flanked by FRT sites, which allow the deletion of the resistance gene by the Flp recombinase after gene replacement. 100ng of the gel purified PCR products were used to electro-transform cells harboring the helper plasmid (pKD46) expressing λ red recombinase. Competent cells were prepared from cultures grown in LB 10 mM L-Arabinose for induction of λ red-recombinase. The recombinants were selected at 37°C on LB chloramphenicol or kanamycin plates, respectively. The loss of the helper plasmid was confirmed by sensitivity to ampicillin and the deletion of the target gene was confirmed by PCR. Two independent colonies were stored in the laboratory strain collection and used in further experiments.

9. Transduction with phage T4GT7 (Wilson et al., 1979)

T4-Topagar
6g Bacto-Agar (Difco)
10g Bacto-Tryptone (Difco)
8g NaCl
2g Tri-Natriumcitrate-Dihydrate
3g Glucose
add 1l H2O

The technique is based on generalized transduction, which makes use of the bacteriophage T4*GT7* to transfer DNA between bacteria. Briefly, 100µl of the overnight culture to be transduced was incubated with 10µl, 5µl, and 2µl of T4GT7 lysate prepared from the cells which had the DNA of interest (Donor strain). The incubation was carried out for 20 minutes at room temperature and 100µl was plated on respective selection plates. The transductants were restreaked at least three to four times to get rid of the contaminating phages and the transfer of the gene was verified by PCR.

10. Integration of plasmids into the *attB* site of the *E. coli* chromosome

Integration of plasmids into the chromosome was done as described (Diederich et al., 1992). Briefly, integrations of originless circularized DNA fragments, containing the *attB* sequence and the spectinomycin cassette are catalyzed by the integrase expressed from a temperature sensitive plasmid with kanamycin resistance gene (pLDR8). The integrants are selected at 42°C, which inhibits the replication of the plasmid. The integrants are screened for Kanamycin sensitivity to ensure the loss of the plasmid. The strain S541 or its derivatives were first transformed with a temperature sensitive plasmid (pLDR8) expressing the integrase, and the transformants were selected at 28°C on LB kanamycin plates. Plasmids carrying the λ *attP* site, the gene *lacZ* fusion of interest and the spectinomycin resistance cassette were digested with BamHI (or BglII). The origin-less fragment was gel purified and eluted using the Qiagen gel extraction kit. 10ng of the origin less fragment was religated and half of the religation was used to transform competent cells of S541/pLDR8. At 37°C the integrase gene is expressed, which promotes recombination between the λ *attB* and *attP* sites resulting in integration of the DNA fragment. The transformants were selected at 42°C on LB spectinomycin plates to select for the integrase catalyzed integration of the DNA fragment into *attB*. In addition, at 42°C replication of the temperature sensitive plasmid pLDR8 stops. The colonies were analyzed for kanamycin sensitivity (loss of pLDR8) and the integration was verified by PCR using the primers mentioned below (primer sequences documented in lab records). Two independent integrants were selected for use in further experiments.

S93/S164: to test the *attB/P`*-side
S95/S96: to test the *attP/B`*-side
S95/S164: to see integrations of dimers

11. β-galactosidase assays

The β-galactosidase activity measurement was carried out essentially as described (Miller, 1972), with only minor modifications. Briefly, strains were grown overnight in 3ml LB with appropriate antibiotics and IPTG (0μM, 20mM 200μM). The subcultures were made in fresh 8mlLB an OD_{600} of 0.15-0.2. The cultures were grown

to an OD_{600} of approximately 0.5 before harvesting. Three different dilutions of culture were made in duplicates in Z-buffer in a final volume of 1ml on ice. The cells were permeablized by addition of 10µl of 0.1% SDS and 20 µl of chloroform. The dilutions were preincubated at 28°C for 10 minutes followed by addition of 200 µl of ONPG (4mg/ml in 0.1M phosphate buffer pH 7.0). The assay was stopped by the addition of 0.5ml 1M Na_2CO_3 and centrifuged. The OD_{420} was measured and the β-galactosidase activity was calculated as described below. The enzyme assays were performed at least three times from independent strains or transformants and the standard deviation was less than 10% unless otherwise indicated.

$$\text{Miller units} = \frac{OD_{420} \times \text{dilution factor} \times 1000}{OD_{600} \times \text{time (minutes)}}$$

12. Preparation of competent cells and transformation ($CaCl_2$ method)

For transformation, the strain of interest was streaked on to a LB plate with suitable antibiotic resistance. A single colony was picked and inoculated overnight in 3ml LB medium with suitable antibiotics and at appropriate temperature. The overnight cells were inoculated in 50ml LB with suitable antibiotics to an OD_{600}=0.05 and grown till OD reaches 0.3. The cells were harvested on ice and transferred to prechilled tubes. The cells were centrifuged at 3000 rpm for 10minutes at 4ºC. The pellets were resuspended in 25ml of ice cold 0.1M $CaCl_2$ and incubated on ice for 20minutes. The cells were pelleted again by centrifugation at 3000 rpm for 10 minutes at 4ºC. The resulting pellet was resuspended in 2ml of ice cold 0.1M $CaCl_2$ and the cells were competent for transformation. For transformation 1-50ng of plasmid DNA or 10µl of ligation reaction was made up to 50µl in TEN buffer and mixed with 100µl of competent cells. The cells were incubated on ice for 20 minutes followed by heat shock at 42ºC for 2 min and additional 10minutes incubation in ice. The competent cells were transferred to 1ml LB medium and incubated for 1hour at 37ºC. 100µl of the culture was plated on suitable selection plates and incubated overnight at 37ºC.

13. SDS-PAGE and Immunoblotting

For the analysis of protein expression by SDS-PAGE and immunoblotting, the overnight cultures carrying respective plasmids were inoculated in 100ml LB with suitable antibiotics to an OD_{600}=0.05 and the cells were grown till OD reached 0.3. The protein expression was induced with 1mM IPTG for 2 hours at 37ºC. In general, 500µl of the cultures (uninduced and induced) were pelleted by centrifugation at 13000 rpm for 5 minutes at room temperature and resuspended in 1x Laemmli buffer to a final concentration of $0.05OD_{600}$ cells per 10µl of Laemmli buffer. 10µl (0.05 OD) of cells were separated on 12% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a SE260 mighty small vertical gel electrophoresis unit (GE Health care). The gels were blotted onto a 0.45µM pore size poly vinylidene difluoride (PVDF) membrane (GE Healthcare) using a TE70 semidry blotting apparatus (GE Healthcare). The blot was handled using standard western blotting protocol (Gallagher et al., 2004). Briefly, for the Westernblotting of the RcsB-HA tagged proteins, a rat-anti-HA immunoglobulin G (IgG) primary antibody (1mg/ml, Sigma) at 1:2000 dilution followed by anti-HA alexaflour®680nm secondary IgG antibody (2mg/ml, Molecular probes) at 1:2000 dilution were used. A mouse-anti-FLAG primary IgG antibody (5mg/ml, Sigma) at 1:5000 dilution followed by anti-mouse alexaflour®680nm secondary IgG antibody (1mg/ml, Li-Cor) at 1:10000 dilution were used for BglJ-FLAG tagged proteins. Finally the blots were visualized using Odyssey imaging system (Li-cor Biosciences) at 700 and 800nm channels and the peak intensity was quantified using Odyssey V1.2 software. The same protocol was followed for Immunoblotting analysis of RcsB-HA and YjjQ-FLAG tagged protein.

14. Co-immunoprecipitation

The following protocol was used for co-immunoprecipitation study of RcsB with BglJ and RcsB with YjjQ in *E. coli* (S3377) Δ*rcsB* Δ*PQJ*). The plasmids expressing RcsB (pKAP38), $RcsB_{D56E}$ (pKEAP43) and $RcsB_{D56N}$ (pKEAP44) were transformed into *E. coli* (S3377) with and with out BglJ (pKERV10). A single colony from each transformation was picked and inoculated overnight in 3ml LB with suitable

antibiotics at 37ºC. The overnight cultures were inoculated in 100ml LB with suitable antibiotics to an OD_{600}=0.05 and grown till OD reached 0.3. The protein expression was induced with 1mM IPTG for 2 hours at 37ºC. An aliquot of uninduced cells kept as controls for SDS-PAGE. (Induction of protein expressions was checked on SDS-PAGE before proceeding to co-immunoprecipitation) The cells were harvested on ice and pelleted by centrifugation at 8000 rpm for 15 minutes at 4ºC (Sorvall, SLA1500). The cell pellet was washed once with 10ml lysis buffer (See materials and methods V.1) and pelleted down again. The resulting pellet was resuspended in 1ml of lysis buffer (pre-chilled). The cells were lysed by sonication at 30% duty cycle, 5 output, 6 to 8 repeats of 10 second pulse with 1minute rest, till the lysate become translucent (Branson sonifier 250 Classic, 3mm micro tip). The samples were kept on ice throughout sonication. The lysate was cleared at 13000 rpm for 15minutes at 4ºC and the supernatant was transferred into a pre chilled 1ml eppendorf tube. The total protein in the lysate was estimated by BCA (Bicinchoninic acid) protein assay (Pierce). For co-immunoprecipitation, 200µg of lysate was made up to 950µl with lysis buffer in a 2ml eppendorf tube for proper mixing. 5µl of rabbit anti-HA IgG antibody (0.6mg/ml, Sigma) was added to the lysates and the samples were incubated for 4 hours in the cold room (4ºC) in a tube rotator. In parallel Protein-A sepharose beads (Protein-A sepharose^{TM} CL-4B, 100mg/ml, GE healthcare) were prepared at 100mg/ml concentration by mixing the slurry in water for 30min followed by incubation in lysis buffer for 2 hours in the cold room in a tube rotator.

For immunoprecipitation 50µl of Protein-A sepharose beads were added to the lysate-antibody complex and incubated for further 2 hours in the cold room in the tube rotator. The protein-antibody-bead complex was pelleted down at low speed at 2000 rpm for 2min at 4ºC. The unbound supernatant was carefully removed (precipitation control) which was analyzed on SDS-PAGE. The beads were washed with 1ml lysis buffer by incubating for 5 min on tube rotator in the cold room and pelleted again. The washing was repeated 3 times and the residual buffer was removed with 7G needle or 0.57mm capillary tips. The beads were mixed with 50µl laemmli buffer and boiled at 95ºC followed by centrifugation at 13000 rpm for 5min at room temperature. The supernatant (immunoprecipitate) was loaded along with lysate on 12% SDS-PAGE and analyzed by Immunoblotting (See Materials and methods VI.13). Similarly

for the co-immunoprecipitation analysis of RcsB with YjjQ, same protocol was followed except BglJ-FLAG tagged plasmid was replaced by a low copy plasmid expressing a C-terminal HA tagged YjjQ.

15. Strep-tag protein purification

For the purification of Strep tagged proteins, *E. coli* strain S3377 (Δ*rcsB* Δ*PQJ*) transformed with plasmid encoding RcsB was grown in 1liter LB_{kan} at 37ºC (28ºC for RcsB $_{D56E}$). The cells were induced at 0.3 OD600 with 1mM IPTG for 2 hours. Aliquots were taken before and after induction for checking expression. The protein expression was always checked before FPLC purification. After 2hrs of induction with IPTG, cells were harvested on ice. Cells were centrifuged at 5000 rpm for 15min at 4°C (Sorvall, SLC6000). The cell pellet was washed with 25ml buffer A and pelleted down again. Finally the cell pellet was resuspended in 10ml buffer A and centrifuged at 4000 rpm for 30min at 4°C (Eppendorf, table top). The cell pellet was stored at -80°C till use. The pellet was resuspended in 10ml buffer A and lysed by EmulsiFlex-C5 (Avestin, Europe GmbH) for 5min under high pressure (10000-15000 psi). The lysate was cleared by Ultra centrifugation (Beckman TL100 Optimax Ultra-centrifuge, MLS50 Swing out rotor) at 20000 rpm at 4°C for 30min. The cleared lysate of ~10ml was transferred to a 15ml tube.

The ÄKTA system pumps were washed with buffer A and B (~ 20ml) respectively. A Strep-tactin superflow column (1ml, Strep-*Tactin*® *Superflow*® *cartridge* H-PR) was equilibrated with 10 column volumes of bufferA. 10ml of filtered lysate was injected through 10ml super loop. The column was washed with 20 column volume of buffer A and the protein was eluted in 500µl fractions with BufferB containing 2.5mM D-desthiobiotin. The fractions were separated on 15% SDS-PAGE, and stained with Coomassie blue at room temperature. The protein concentration of the purest fractions were measured by Bradford (Biorad Protein assay) and dialyzed using a Float-A-lyzer with a 5kDa cutoff (Spectra/Por® Float-A-Lyzer® G2, Spectrum labs) with 4 changes of dialysis buffer for every 60 minutes in cold room. The dialyzed fractions were aliquoted and stored at -80°C. For regeneration, the column was washed with 10CV buffer A, followed by 15 column volume of buffer R containing 1mM HABA (2-[4'-hydroxy-benzeneazo] benzoic acid). The red colored isomer forms a complex at

the biotin-binding site of strep-tactin. The color change of the column bed to red color indicates completion of regeneration. HABA is removed by washing with 40 column volume of buffer A, till the column color turns to white, which is the indication of column is ready for use again.

16. FLAG-tag protein purification

For the FLAG tagged protein purification, an *E. coli* strain (S3377 Δ*rcsB* Δ*PQJ*) expressing BglJ plasmid was grown in 1liter LB_{kan} at 37ºC. The cells were induced at 0.3 OD600 with 1mM IPTG for 2 hours. Aliquots were taken before and after induction for checking expression (the protein expression was always checked before purification). After 2 hrs induction with IPTG, the cells were harvested on ice and centrifuged at 5000 rpm for 15min at 4°C (Sorvall, SLC6000). The cell pellet was washed with 25ml buffer A and pelleted down again. Finally the cell pellet was resuspended in 10ml buffer A and and centrifuged at 4000 rpm for 30min at 4°C (Eppendorf, table top). The pellet was resuspended in 8ml buffer A and lysed by EmulsiFlex-C5 (Avestin, Europe GmbH) for 5 min under high pressure (10000-15000 psi). The lysate was cleared by Ultra centrifugation (Beckman TL100 Optimax Ultra-centrifuge, MLS50 Swing out rotor) at 20000 rpm for 30min at 4°C. 1ml of the cleared lysate was mixed with 100µl of FLAG beads (Sigma, EZview Red ANTI-FLAG M2 affinity gel) for 3 hours in cold room. The tube was centrifuged at 1000rpm for 1min at 4°C and unbound was collected (should not exceed 2000rpm). The beads were washed 3 times with 500µl of buffer B (The beads were transferred into a new tube during each wash) and centrifuged at 1000rpm for 1min at 4°C. The Strep-tagged protein elution, 100µl of buffer A containing 300µg FLAG peptide (Sigma) was incubated for 2hours at cold room in a tube rotator. The tube was centrifuged at 1000rpm for 1minute at 4°C followed by 1 minutes spin at maximum speed. The purified elute was checked on 15% SDS-PAGE and confirmed by western blotting using mouse anti-FLAG/anti-mouse alexaflour® 680nm antibody. The purified elute was dialyzed using a Float-A-lyzer with a 5kDa cutoff (Spectra/Por® Float-A-Lyzer® G2, Spectrum labs) with 4 changes of dialysis buffer for every 60 minutes in cold room. The dialyzed fractions were aliquoted and stored at -80°C.

17. Electrophoretic mobility shift assay (EMSA)

For the mobility shift assay, the DNA fragments (*bgl, osmC, lacZ)* were PCR amplified and gel purified. The concentration of each DNA fragment was adjusted to 30ng/µl. The DNA fragments were diluted in EMSA binding buffer and 15ng of DNA fragment was used per assay (All three DNA fragments were used for every assay). The DNA fragments were incubated with increasing concentration of purified H-NS (0µM to 1.6µM) and RcsB proteins (0µM to 1µM) with constant amount of partially purified BglJ protein (2µl). The 10µl EMSA reaction volume to (usually 2µl DNA fragments are mixed with (upto 8µl) protein mix and final volume is made up to 10µl with binding buffer). The reaction mix was incubated at 30ºC for 20minutes in heating block. The protein DNA complex was separated on 8% (29:1) non-denaturing polyacrylamide gel which was pre run for 20 minutes in 0.5x TBE. Each well was loaded with 6µl of 0.5x DNA loading dye for visualization. The 10µl of the EMSA reaction mix was loaded along the side walls of the well. The reaction mix displaces the dye and settles down at the bottom of the well. The gel was run at 200V for approximately 90 minutes in 0.5x TBE in cold room. The gel was stained with ethidium bromide (0.5µg/ml) for 20 minutes at room temperature.

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