INTERFERING WITH REGULATORS OF BIOFILM FORMATION

A NOVEL BIOFILM INHIBITORY STRATEGY

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Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Bio-ingenieurswetenschappen

December 2015

Doctoraatsproefschrift nr. 1322 aan de faculteit Bio-ingenieurswetenschappen van de KU Leuven

Uitgegeven in eigen beheer, Elke Van Assche, Leuven

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DANKWOORD

Met het schrijven van dit dankwoord ben ik bijna op het einde van mijn doctoraatsopleiding aanbeland. Hoewel ik met veel vertrouwen en vol goede moed was begonnen aan dit project, liep niet alles zoals ik vooraf had gepland of gehoopt. Dat ik vandaag toch in de finale fase zit, is mede dankzij de hulp en de steun van heel veel mensen die ik daar op deze manier heel graag voor zou willen bedanken.

In de eerste plaats wil ik mijn promotoren, Prof. Jos Vanderleyden en Dr. Hans Steenackers bedanken. Jos, bedankt voor de kansen die u mij hebt gegeven, de tijd die u maakte om te brainstormen over mijn project, uw hulp bij het nalezen en uw goede raad bij moeilijke beslissingen. Hans, een beetje onverwacht ben je in de loop van mijn doctoraat mijn co-promotor geworden, maar zoals je zelf ooit hebt gezegd, is mijn doctoraat uiteindelijk een beetje op het jouwe gaan lijken. Bedankt voor de kansen die je mij gegeven hebt en voor het nalezen van mijn teksten. Ik bewonder je enorme werklust en je wetenschappelijk enthousiasme.

Thanks to the chairman and the members of my examination committee for their time and effort and for thoroughly reading this manuscript. Your valuable comments enabled me to improve the quality of this work.

Mijn volgend woordje van dank gaat naar mijn collega's. Sandra, bedankt voor alle MicA brainstorms en voor je hulp en goede raad bij het schrijven van mijn artikel en thesis. Ook heel erg bedankt om de RNA-seq data te analyseren. Sigrid en Gwendoline, jullie hulp en enthousiasme was van groot belang bij de start van dit project. Ik heb enorm veel van jullie geleerd, dank je wel. David, bedankt om mij wegwijs te maken in de wondere wereld van RNA-isolaties, northern blots en qRT-PCR's en voor je hulp bij de experimenten. Ami en Tine, bedankt voor jullie hulp en voor de leuke babbels als ik even een momentje van ontspanning nodig had. Stefanie, bedankt voor je steun en de babbels tussendoor, vanaf onze gezamelijke start aan het CMPG tot nu. ElkeL, Ansie en Stijn, mijn bureaugenootjes, bedankt voor jullie steun en motivatie tijdens deze laatste maanden. Het was heel

leuk dat jullie er waren als ik even een dipje had. Ilse, bedankt voor al je werk en voor de leuke en leerrijke tijd tijdens je masterthesisjaar.

Daarenboven wil ik alle bovengenoemde personen samen met Lise, Seppe, Mariya, Elien, Kenny, Irina, Lyn, Marijke, Hanne, Cynthia en alle andere oud-S&P'ers, (oud-)SPI'ers en (oud-)PFI'ers bedanken voor hun interesse en voor de leuke sfeer in het labo, tijdens middagpauzes, tijdens de sportcompetities, tijdens quizzen, closing-times, kerstfeestjes en recepties. Dankzij jullie ben ik de voorbije jaren elke dag met heel veel plezier komen werken.

Anita, heel erg bedankt voor al je hulp bij de administratieve uitdagingen die ik tijdens mijn periode hier ben tegengekomen. Een woordje van dank ook voor JosD en André. Dankzij jullie drieën heb ik in een geweldig goed georganiseerd labo kunnen werken.

Mijn huisgenootjes Anneke, Birgit, Ineke en Sanne; en Dieter en Anneleen, bedankt voor jullie steun, voor alle wetenschappelijke en vooral alle iets minder wetenschappelijke gesprekken en voor de gezellige en ontspannende avonden in Heverlee.

Anne, Dirk, Jelle, Ellen, SaraL, SaraV, SaraP, het is jullie waarschijnlijk nog steeds een raadsel wat ik hier de voorbije jaren heb gedaan. Toch bedankt voor de interesse, de gezellige autoritjes naar en vanuit Kapelle-op-den-Bos en de wekelijkse muzikale ontspanningsmomenten.

Tot slot, moeke en vake, bedankt voor de kansen die jullie mij gegeven hebben, de interesse in mijn werk en alle hulp en verwennerij daarbuiten.

SUMMARY

Natural bacterial populations predominantly grow in biofilms. This is a structured community of cells, which are attached to a surface and/or to each other and are embedded in a self-produced polymeric matrix. Bacteria in a biofilm have an increased tolerance against antibiotics, disinfectants and components of the immune system. This makes it difficult to remove detrimental biofilms, for example on medical implants, surfaces in the food-processing industry or in industrial pipelines. Therefore, there is a need for molecules that prevent biofilm formation. Because of the increasing knowledge about the different processes that take place and the cellular components that are necessary when a biofilm is developing, target-based anti-biofilm molecules can be designed. In this way, studies already identified anti-biofilm molecules that specifically prevent attachment or destabilize the matrix.

Increasing knowledge is also available about the regulators involved in biofilm development. Biofilm formation is indeed an energy-consuming and complex process that requires tight regulation. Consequently, we speculated that interfering with the activity of important biofilm regulators, using the knowledge about the mode of action and the binding requirements of these molecules, might prevent or delay the formation of a mature biofilm. More specifically, the post-transcriptional regulators of biofilm development are of special interest. The sRNAs and RNA-binding proteins that belong to this class mainly regulate target gene expression by affecting mRNA stability or translation efficiency through interaction with mRNA molecules. Therefore, it should be possible to impair the activity of these regulators with complementary RNA sequences or RNA decoys.

The hypothesis that RNA sequences can be used to target post-transcriptional regulators and that this can disturb biofilm development, was validated using the CsrA-based regulatory network, which is known to be involved in the regulation of biofilm formation. CsrA is the central global regulatory protein, of which the activity is regulated by the sRNAs CsrB, CsrC and McsA. Both interfering with

the activity of CsrA and CsrB was evaluated and resulted in the identification of a decoy sequence that was able to decrease the activity of CsrA and reduce the biofilm forming ability of strains that express this sequence. Furthermore, we were able to show that a synthetic PNA-based analog of this sequence also has sequence-specific anti-biofilm activity, suggesting that this is novel and promising anti-biofilm strategy. On the other hand, interfering with CsrB activity using a complementary sequence did not have an effect on biofilm development.

Another interesting RNA sequence that reduced biofilm formation when it was expressed in the cell was not involved in targeting CsrA or CsrB. An RNA-seq analysis showed that in the presence of this sequence, the expression of curli and motility genes was downregulated and the expression of genes necessary to survive under low pH was upregulated. Furthermore, a network analysis of the expression data suggested that the transcription regulators H-NS or Lrp might be the target of this sequence. Although their identification as a true target requires further validation, this indicates that RNA sequences might also be used to interfere with the activity of some DNA-binding regulatory proteins.

Additionally, an alternative mechanism to control the regulatory activity of the sRNA MicA was studied. Although it could not be confirmed that *metC* is regulating MicA stability, this natural regulatory mechanism might be an inspiration source for the development of RNA sequences that destabilize biofilm regulating sRNAs, broadening the applicability of nucleic acid-based anti-biofilm molecules.

In conclusion, the experiments in this PhD thesis confirmed that RNA sequences can reduce the biofilm forming capacity of a strain. Synthetic analogs of these sequences are therefore promising biofilm inhibitors. They can probably not only be used for the strategy that was validated here, targeting post-transcriptionally active RNA-binding proteins using a decoy approach, but might also be interesting to reduce the activity or stability of DNA-binding proteins or sRNAs in the future.

SAMENVATTING

Natuurlijke populaties van bacteriën komen vooral voor als biofilmen. Dit zijn gestructureerde gemeenschappen van cellen die aangehecht zijn aan een oppervlak en/of aan elkaar en omgeven zijn door een zelfgeproduceerde matrix van polymeren. Bacteriën die in een biofilmstructuur groeien hebben een verhoogde tolerantie voor antibiotica, desinfectiemiddelen en componenten van het immuunsysteem. Hierdoor zijn biofilmen die problemen veroorzaken, bijvoorbeeld op implantaten, op oppervlakken in de voedselverwerkende industrie of in pijpleidingen, moeilijk te verwijderen. Daarom is er nood aan moleculen die biofilmvorming verhinderen. Omdat de kennis over de verschillende processen die plaatsvinden en de celcomponenten de nodig zijn bij biofilmvorming steeds toeneemt, kunnen doelwit-gebaseerde anti-biofilm moleculen ontwikkeld worden. Op die manier werden in eerdere studies reeds anti-biofilm moleculen geïdentificeerd die specifiek aanhechting verhinderen of de matrix destabiliseren.

Er is ook steeds meer kennis over de regulatoren die betrokken zijn bij de ontwikkeling van een biofilm. Biofilmvorming is inderdaad een complex proces dat veel energie vraagt en dus strikt gereguleerd moet worden. Daarom gaan we ervan uit dat door te interfereren met de activiteit van belangrijke biofilmregulatoren, gebruikmakend van de kennis over hun werkingsmechanisme en hun bindingsvereisten, de vorming van een mature biofilm verhinderd of vertraagd kan worden. Meer specifiek wekken vooral de post-transcriptionele regulatoren interesse. De sRNAs en RNA-bindende eiwitten die tot deze klasse behoren, reguleren de genexpressie door de stabiliteit en de translatie-efficiëntie van mRNAs te beïnvloeden door hieraan te binden. Daarom zou het mogelijk moeten zijn om de activiteit van deze regulatoren te verhinderen met behulp van complementaire RNA sequenties of RNA ‘decoys’.

De hypothese dat RNA sequenties gebruikt kunnen worden om post-transcriptionele regulatoren te targeten en dat dit het proces van biofilmvorming, wat gereguleerd wordt door een aantal van deze

regulatoren, verstoort, werd gevalideerd op basis van het CsrA-gebaseerde regulatorisch netwerk. Hiervan is geweten is dat het betrokken is bij biofilmvorming. CsrA is het centrale regulatorisch eiwit waarvan de activiteit gereguleerd wordt door de sRNAs CsrB, CsrC en McaS. Zowel interfereren met CsrA als met CsrB werd geëvalueerd en resulteerde in de identificatie van een 'decoy' sequentie waarmee de activiteit van CsrA verminderd kon worden en waarmee ook biofilmvorming gereduceerd kon worden. Daarenboven werd aangetoond dat een PNA-gebaseerd synthetisch analoog van deze sequentie ook sequentie-specifieke anti-biofilmactiviteit had, wat suggereert dat dit een nieuwe en veelbelovende anti-biofilmstrategie is. Interfereren met de activiteit van CsrB had geen effect op biofilmvorming.

Een andere interessante RNA sequentie die biofilmvorming verhinderde had niet CsrA of CsrB als doelwit. Een RNA-seq analyse toonde aan dat wanneer de sequentie tot expressie kwam, de expressie van de genen betrokken bij de productie van curli en bij motiliteit onderdrukt werd en de expressie van de genen nodig om te overleven bij lage pH gestimuleerd werd. Een netwerkanalyse suggereerde dat de sequentie H-NS en Lrp activiteit mogelijk verminderde, wat doet vermoeden dat RNA sequenties ook gebruikt kunnen worden om te interfereren met de activiteit van sommige DNA-bindende regulatorische eiwitten.

Daarnaast werd ook een alternatief mechanisme om de activiteit van het sRNA MicA te controleren, bestudeerd in dit doctoraatsproject. Hoewel de voorspelde regulatie van de MicA stabiliteit door *metC* niet gevalideerd kon worden, kan dit natuurlijke regulatorisch mechanisme een inspiratiebron zijn voor de ontwikkeling van RNA sequenties die de stabiliteit van biofilm regulerende sRNAs verminderen, wat de toepasbaarheid van nucleïnezuur-gebaseerde anti-biofilm moleculen nog vergroot.

Samenvattend bevestigen de experimenten in dit doctoraatsonderzoek dat RNA sequenties de capaciteit om een biofilm te vormen kunnen verminderen. Synthetische analogen van deze sequenties zijn daarom veelbelovende biofilminhibitoren. Ze kunnen gebruikt worden om de activiteit van RNA-bindende eiwitten betrokken bij post-transcriptionele regulatie te targeten met 'decoy' moleculen, wat hier werd gevalideerd. Daarenboven kunnen ze ook interessant zijn om in de toekomst de activiteit of stabiliteit van sommige DNA-bindende eiwitten of sRNAs te reduceren.

LIST OF ABBREVIATIONS

Ap^R	Ampicillin resistant
Bp	Base pairs
BF	Biofilm phase
CDS	Coding sequence
CFA	Colony-forming antigen
CFU	Colony forming units
CPP	Cell-penetrating peptide
EPS	Extracellular-polymeric substances
GO	Gene ontology
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB	Luria-Bertani
LNA	Locked nucleic acid
MOCO	Molybdenum cofactor
OD	Optical density
PBS	Phosphate buffered saline
PGA	Poly-N-acetylglucosamine
PL	Planktonic phase
PMO	Phosphorodiamidate morpholino oligomer
PNA	Peptide nucleic acid

qRT-PCR	Quantitative reverse transcriptase – polymerase chain reaction
RBP	RNA-binding protein
RBS	Ribosome binding site
RNA-Seq	RNA-sequencing
RS	Restriction site
SELEX	Systematic evolution of ligands by exponential enrichment
SL	Stem loop
SLS	Stem loop stem
sRNA	Small non-coding regulatory RNA
TRAP	trp RNA-binding attenuation protein
TSB	Tryptic Soy Broth
UTR	Untranslated region

CHAPTER 1

INTRODUCTION

General introduction and outline of the thesis

In this first chapter, a general introduction on biofilm development and the regulators that control this process in *Escherichia coli* is given, with special emphasis on the post-transcriptional regulators that are involved. Subsequently, biofilm inhibitors that were developed based on the knowledge about processes involved in biofilm development, are described. In the final part of this chapter, the outline and objectives of this thesis are discussed.

1.1. Biofilm development and anti-biofilm molecules

In natural environments, bacteria predominantly grow in biofilm structures. These are dense, multicellular communities, which are embedded in a self-produced polymeric matrix and are attached to a surface and/or to each other. Biofilm cells differ from free-living cells amongst others by slower growth and metabolism, a higher number of cells in a dormant persister state and an elevated stress response. Additionally, gradients of oxygen are present along the biofilm structure and a matrix is surrounding the cells (Stewart, 2002). These typical characteristics contribute to the increased tolerance of biofilm cells against physical stresses, antimicrobials, disinfectants and components of the immune system (Høiby *et al.*, 2010). Therefore, detrimental biofilms, such as biofilms of pathogenic bacteria on tissues or implants that cause chronic or device-related infections, biofilms of foodborne pathogens on surfaces in food packaging or food processing industry that cause cross-contamination and biofilms of non-pathogenic bacteria that cause corrosion of a surface or reduce the efficiency of industrial processes, can be extremely difficult to remove and result in significant medical threats and economic losses. Consequently, there is a need for molecules that prevent biofilm formation. These anti-biofilm molecules can then be used alone or in combination with conventional antimicrobials or disinfectants to remove bacterial populations. Although a blind high-throughput screening can identify molecules with anti-biofilm activity (Junker & Clardy, 2007; Musk *et al.*, 2005; Ren *et al.*, 2005; Robijns *et al.*, 2012), the increasing knowledge about biofilm formation and the regulators involved in this process enables a more rational design of anti-biofilm molecules. Therefore, in the following section, both the processes and the regulators involved in biofilm development in *Escherichia coli* will be discussed.

1.1.1 Biofilm development

E. coli can form different kinds of biofilms on a variety of surfaces, including submerged biofilms on plastic and glass surfaces, floating pellicle biofilms at an air-liquid interface and macrocolonies on agar plates (Danese *et al.*, 2000; Hung *et al.*, 2013). In general, the development of a biofilm occurs in different stages, comprising the initial attachment of cells to the surface, the formation of microcolonies by cell-cell adhesion, maturation of the biofilm, which coincides with the formation of the extracellular matrix and finally, detachment and dispersion of planktonic cells from the biofilm structure (Stoodley *et al.*, 2002). During the initial attachment stage, reversible interactions take

place between (motile) cells and a favourable surface. These are mainly dependent on electrostatic interactions between the outer membrane of the bacteria and the surface to which they attach. Additionally, attachment is facilitated by adhesive organelles such as pili and curli. During this early stage of biofilm development, motility is necessary in some *E. coli* strains (Guttenplan & Kearns, 2013). In the following maturation stage, the bacteria-to-surface interactions become irreversible and cells in the biofilm start to grow in a multi-layered three-dimensional structure forming differentiated, mushroom- or pilar-like structures interspersed with fluid-filled channels (Stoodley *et al.*, 2002). The formation of these three-dimensional structures is stabilized by bacteria-bacteria interactions, which are facilitated by adhesins like Ag43. At this stage, the bacteria also become surrounded by an extracellular matrix that consists of mostly water, cells, ions and extra-polymeric substances (EPS). These EPS are predominantly composed of polysaccharides, but also contain proteins, nucleic acids (mainly extracellular DNA), lipids/phospholipids, nutrients and metabolites (Donlan, 2002; Flemming & Wingender, 2010; Flemming *et al.*, 2007; Hobley *et al.*, 2015; Sutherland, 2001). The polysaccharides composing the EPS in *E. coli* are generally poly-N-acetylglucosamine (PGA), colanic acid and cellulose (Römling & Galperin, 2015; Wang *et al.*, 2004). The protein fraction of the EPS includes adhesins and other cell surface associated proteins like flagella and curli (Martínez & Vadyvaloo, 2014). In the final phase of biofilm formation, detachment and dispersion takes place. At this stage, the surface attached communities give rise to planktonic cells that can colonize new surfaces.

Together with the identification of the processes active in the different stages of biofilm development, there is increasing knowledge about the regulators that are involved. The switch between planktonic and surface-associated mode of growth is indeed tightly controlled, as the synthesis and the assembly of a biofilm and especially the formation of the matrix, is an extremely energy consuming process (Chambers & Sauer, 2013). Therefore, alternative sigma factors, two-component systems, small RNAs (sRNAs), regulatory RNA-binding proteins and second messengers (especially c-di-GMP) (Mika & Hengge, 2014) integrate different environmental signals and only activate genes necessary for biofilm development under the appropriate environmental conditions. Once biofilm formation is initiated, the formation of a mature biofilm additionally requires tight regulation, enabling the coordinated expression and simultaneous regulation of the many different genes involved in the process. Therefore, complex regulatory networks regulate the spatial and temporal expression of the genes involved in biofilm development. These regulatory networks include both transcriptional and post-transcriptional regulatory elements. Some important

transcriptional regulators will be described, but particular emphasis will be placed on the post-transcriptional regulators involved in biofilm development.

1.1.2 Regulators of biofilm development

The transcriptional regulators RpoS, YdaM and CsgD are key factors in the regulatory network that controls biofilm formation in *E. coli* (Mika & Hengge, 2014). RpoS, which functions as the alternative sigma factor σ^S, is the master regulator of the general stress response. RpoS is well-known for its role in stationary growth phase, where the sigma factor promotes transcription of genes necessary to cope with limitation of nutrients and accumulation of waste products. Under biofilm growth, which is also a stressful growth condition because of intense nutrient competition and the presence of harmful metabolites, RpoS is necessary as well. Its activity is essential for the production of the matrix components cellulose and curli, through the transcriptional activation of the genes encoding CsgD and YdaM (see **Figure 1.1**). CsgD directly activates transcription of the genes responsible for the production and the export of curli, encoded by the *csgBAC-csgDEFG* operons. Additionally, CsgD regulates the formation of cellulose by activating *adrA*, also named *yaiC*, encoding a diguanylate cyclase that synthesizes c-di-GMP, and *yoaD*, a phosphodiesterase that degrades c-di-GMP (Brombacher *et al.*, 2006). Together they cause subtle modifications in c-di-GMP concentrations. c-di-GMP subsequently allosterically stimulates the production of cellulose, modulating the enzymatic activity of cellulose synthase (BcsA-BcsB) (Morgan *et al.*, 2014). YdaM, on the other hand, is a transcriptional co-activator and a diguanylate cyclase that, together with MlrA, activates *csgD* transcription. Additionally, *csgD* expression is regulated by other transcription regulators, including the activators OmpR and IHF and the repressors H-NS, CpxR and RstA (Ogasawara *et al.*, 2010).

At the post-transcriptional level, the global regulatory RNA-binding protein CsrA is an important biofilm regulator. In most cases, this protein post-transcriptionally regulates target gene expression by competing with ribosome binding, which reduces translation initiation efficiency. This often coincides with reduced mRNA stability, as mRNAs that are not bound by ribosomes are less protected from ribonuclease cleavage (Deana & Belasco, 2005). However, for this regulatory protein, other working mechanisms have also been described, which will be discussed in detail in **Chapter 2**. CsrA is called the 'evil twin' of RpoS, as the protein represses stationary phase gene expression and activates genes necessary for exponential growth. In accordance with its opposing activity compared to RpoS, *csrA* expression has been shown to repress biofilm formation (Jackson *et al.*, 2002). The

most important target of CsrA that is responsible for its role in biofilm development is the *pgaABCD* operon. The proteins encoded by these genes are required for the synthesis and the secretion of PGA, one of the matrix composing polysaccharides responsible for attachment, cell-cell adherence and stabilization of the biofilm structure (Wang *et al.*, 2004, 2005). CsrA directly blocks translation of the *pgaABCD* operon and consequently, PGA production is only possible in the absence of bound CsrA. Additionally, CsrA has recently been implicated in the transcriptional regulation of *pgaA* by making a transcription terminator site available when CsrA is bound (Figueroa-Bossi *et al.*, 2014). CsrA has also an indirect effect on PGA synthesis by targeting the transcriptional activator of the *pga* operon, NhaR. CsrA blocks translation of NhaR, resulting in the repression of *pgaABCD* transcription (Pannuri *et al.*, 2012). As CsrA also targets the *glgCAP* operon (Liu *et al.*, 1995; Romeo *et al.*, 1993), involved in the synthesis and turnover of glycogen, which generates precursors of PGA, CsrA also indirectly regulates PGA synthesis, and consequently biofilm formation, in an additional way.

Besides regulating PGA synthesis, CsrA is involved in regulating other processes that are related to biofilm development. Firstly, CsrA plays a role in the regulation of the c-di-GMP metabolism by regulating the expression of two diguanylate cyclases responsible for the synthesis of c-di-GMP, *ycdT* and *ydeH* (Jonas *et al.*, 2008). In general, elevated c-di-GMP levels lead to biofilm formation, while reduced c-di-GMP levels decrease biofilm formation. This can be attributed, amongst other factors, to the allosteric stimulation of cellulose production by c-di-GMP, as already mentioned. Secondly, CsrA binds and stabilizes the *flhDC* transcript, whereby it promotes motility (Wei *et al.*, 2001; Yakhnin *et al.*, 2014). Although motility is required in the initial steps of biofilm development in some *E. coli* strains, promoting motility generally opposes efficient biofilm formation (Guttenplan & Kearns, 2013). A last CsrA target involved in the regulation of biofilm development is *hfq* (Baker *et al.*, 2007). The Hfq protein is involved in the regulation of a multitude of processes, and has different functions. One of its functions is the stabilization of sRNAs and the promotion of sRNA-mRNA interactions. Hfq is thought to regulate biofilm formation through this role in sRNA-dependent regulation, as different sRNAs are shown to be important post-transcriptional regulators of genes involved in biofilm development (Martínez & Vadyvaloo, 2014; Mika & Hengge, 2014).

sRNAs were originally described as short (150-500 base pairs) non-coding single stranded RNA molecules that are not translated into proteins unlike transcription factors, thereby reducing time and energy compared to transcription factor-based regulation. However, multiple sRNAs that deviate from this definition have been described (Liu & Camilli, 2010). Most sRNAs have a post-transcriptional effect on gene expression by interacting with their target mRNAs through direct antisense pairing (Liu & Camilli, 2010). They typically bind at or near the RBS of their mRNA target

which affects translational efficiency by competing with ribosome binding (Bouvier *et al.*, 2008), although sRNAs binding more upstream or downstream of the RBS have been described (Desnoyers & Masse, 2012; Pfeiffer *et al.*, 2009; Sharma *et al.*, 2007). sRNA binding to its target can also have a positive effect on gene expression, as some sRNAs stabilize the transcript or open an inhibitory secondary structure that occludes the RBS (Papenfort & Vanderpool, 2015; Soper *et al.*, 2010). Other sRNAs bind proteins instead of mRNAs and act by sequestering this protein, thereby inhibiting protein activity (Göpel *et al.*, 2013; Gottesman & Storz, 2011; Liu *et al.*, 1997). Most sRNAs identified today are *trans*-encoded sRNAs. These share only limited complementarity with their target (10-25 bp) and can consequently have multiple targets. This enables the sRNA to regulate the expression of different genes in a regulatory network. Moreover, one mRNA target can be regulated by multiple sRNAs, whereby target gene expression can be fine-tuned according to different signals.

Different sRNAs involved in biofilm development regulate the expression of some of the regulatory proteins that were discussed above, such as RpoS, YdaM, CsgD and CsrA. The sRNA-dependent post-transcriptional regulation of their expression comes on top of the more generally accepted transcriptional regulation and adds an extra layer of complexity to the regulatory process. Three different sRNAs, ArcZ, DsrA and RprA, activate *rpoS* translation by binding to a region upstream of the RBS, which results in opening an inhibitory structure that occludes the RBS and makes the translation of RpoS inefficient (Lease & Woodson, 2004; Majdalani *et al.*, 1998, 2002; Mandin & Gottesman, 2010). The expression of these different *rpoS* regulating sRNAs is induced under different environmental conditions: *dsrA* expression is induced under cold temperatures (Repoila & Gottesman, 2001, 2003), *rprA* expression is induced under cell envelope stress (Majdalani *et al.*, 2002), while *arcZ* expression is activated under aerobic growth (Mandin & Gottesman, 2010). ArcZ is the most important sRNA regulator of *rpoS* expression since its deletion leads to lower RpoS levels than deletion of the other sRNAs. One identified sRNA, OxyS, the expression of which is induced under oxidative stress (Altuvia *et al.*, 1997), inhibits the translation of *rpoS*. Possibly its effect is caused by a competition with other sRNAs for Hfq binding. As Hfq is necessary for the stability and the activity of most sRNAs, the presence of high levels of OxyS might indeed result in lower levels of active sRNAs that are able to promote RpoS translation (Zhang *et al.*, 1998).

Besides sRNAs regulating *rpoS* expression, there are also sRNAs that target *csgD* and/or *ydaM*, thereby influencing biofilm development. Some sRNAs target both transcription factors in the cascade, which causes a direct and an indirect effect on *csgD* expression (Mika & Hengge, 2014). RprA, which was already described as it positively regulates *rpoS* expression, negatively regulates *csgD* and *ydaM* expression (Mika *et al.*, 2012). Although the fact that this sRNA acts positively on

rpoS expression and negatively on targets more downstream in the regulatory cascade seems to be somewhat counteracting, these interactions occur with different sensitivities, with the strongest interaction between RprA and the *csgD* transcript. McaS is another sRNA that negatively influences *csgD* expression. Its expression is activated by cAMP-CRP in response to carbon limitation (Thomason *et al.*, 2013). Finally, OmrA and OmrB also target *csgD* and *ydaM*. These two sRNAs are activated under conditions of high osmolarity by the EnvZ/OmpR two component system (Guillier & Gottesman, 2006). Both sRNAs are encoded in the genome next to each other, are highly similar and have the same mRNA targets (Guillier & Gottesman, 2008; Wassarman *et al.*, 2001). In contrast to RprA and McaS, OmrA and OmrB do not reduce *csgD* mRNA levels but only affect translation efficiency (Holmqvist *et al.*, 2010). *gcvB*, activated in response to high amino acid levels, also reduces *csgD* expression (Jørgensen *et al.*, 2012; Mika *et al.*, 2012). However, it is not clear whether this is via *rpoS*, *csgD* or another unidentified target that might influence *csgD* expression (Mika & Hengge, 2014). The last sRNA that has recently been shown to downregulate *csgD* expression is RydC (Bordeau & Felden, 2014). For an overview of sRNAs regulating *rpoS*, *ydaM* and *csgD*, see **Figure 1.1**.

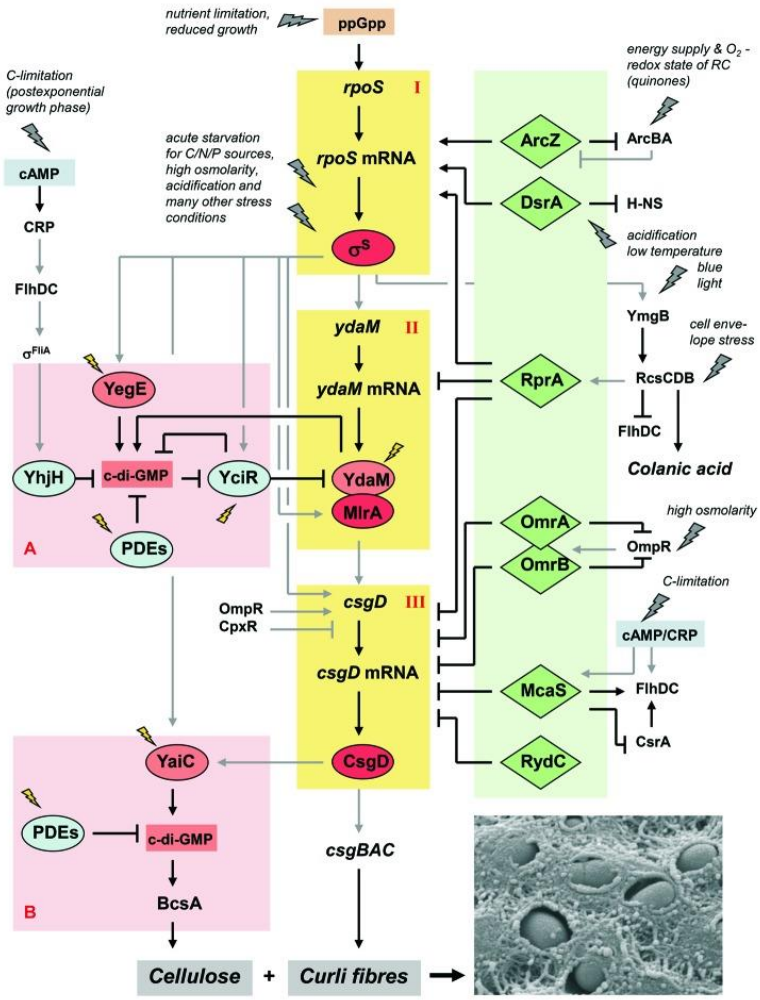


Figure 1.1 Signal integration in the RpoS-dependent network that regulates the synthesis of the extracellular matrix components curli and cellulose. The "backbone" of the control network is a transcription factor cascade consisting of three modules (I, RpoS as the master regulator; II , YdaM/MlrA; III , CsgD; highlighted by yellow boxes). A c-di-GMP-dependent control module (A; with diguanylate cyclases (DGCs) and phophodiesterases (PDEs) highlighted by light red and light blue ovals, respectively), with the PDE YciR acting as a trigger enzyme, provides an essential switch between transcription factor modules I and II . A second c-di-GMP control module (B) allows for specific signal input into cellulose production without affecting curli expression. sRNAs (highlighted in green) provide for environmental input along the entire transcription factor cascade, with *rpoS* mRNA and *csgD* mRNA acting as major hubs for signal integration. Only sRNAs are included for which direct binding at the respective mRNAs was demonstrated by compensatory base pair exchanges. For simplicity, the reverse action is not included in the figure, i.e., that high production of these mRNA, e.g. during entry into stationary phase, can scavenge the sRNAs, and thereby, affect their other regulatory effects. Activation of gene expression by transcription factors is depicted by gray arrows, all other regulatory effects are symbolized by black arrows or lines (Mika & Hengge, 2014).

Quite a number of sRNAs that influence the *rpoS-ydaM-csgD* regulatory cascade, also affect the expression of *flhDC*, which is the master regulator of motility. Motility is necessary for initial attachment, but needs to be reduced once attachment has taken place as motility opposes efficient biofilm formation. Therefore, there is often a negative correlation between the regulation of *flhDC* and *csgD* expression, illustrated by the repressive activity of CsgD on flagellar operons and by the action of the biofilm regulating sRNAs influencing both regulons. In relation to this sRNA-dependent regulation, ArcZ and DsrA, two sRNAs that activate *rpoS* translation and consequently activate *csgD* expression, negatively affect, directly or indirectly, the expression or stability of the *flhDC* master regulator. The same opposing effect on *csgD* and *flhDC* expression is observed for OxyS and McaS (De Lay & Gottesman, 2012). Remarkably, OmrA and OmrB downregulate both *csgD* and *flhDC* expression. A possible explanation for this rather unusual coupling between the expression regulation of these transcription factors is that OmrA and OmrB are activated under conditions of high osmolarity, where both the production of flagella and curli are deleterious (Mika & Hengge, 2014).

Other sRNAs that play a role in the regulation of biofilm development regulate the activity of the regulatory RNA-binding protein CsrA. CsrB and CsrC are the two best studied sRNAs with this activity. They both have multiple regions that resemble CsrA binding sites, thereby binding CsrA with high affinity and inhibiting is regulatory activity (Liu *et al.*, 1997). CsrB and CsrC have redundant functions, although the number of CsrA binding sites is lower for CsrC (Weilbacher *et al.*, 2003). *csrB* and *csrC* expression is controlled by the BarA-UvrY 2-component system (Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003) and their expression is induced when cells encounter poor nutrient conditions, oxidative stress and perturbations in the Krebs cycle (Jonas & Melefors, 2009). Their stability is not dependent on Hfq, but is influenced by CsrD, which recruits the sRNA to RNaseE and promotes its degradation (Suzuki *et al.*, 2006). CsrD was thus far not linked to the regulation of the stability of other sRNAs. McaS, a sRNA that was already mentioned earlier as it regulates the expression of *csgD* and *flhDC*, the respective master regulators of biofilm formation and motility, also bears CsrA binding sequences, whereby the sRNA can sequester CsrA, similar to CsrB and CsrC. However, the number of CsrA binding sites is low compared to the number of binding sites in CsrB (Jørgensen *et al.*, 2013). It is likely that these CsrA-regulating sRNAs are expressed under different growth conditions.

Besides targeting *rpoS*, *csgD*, *flhDC* or CsrA, *E. coli* sRNAs are involved in the regulation of biofilm development in other ways. For example, another transcriptional regulator of biofilm formation, H-NS, interferes with the expression of *rpoS*-dependent genes through competing with promoter binding (Belik *et al.*, 2009; Hengge-Aronis, 2002). The expression of this protein is negatively

regulated by the sRNA DsrA, which is also involved in *rpoS* regulation (Lease *et al.*, 1998; Majdalani *et al.*, 2005). Additionally, it is likely that a lot of biofilm regulating sRNAs are still unknown.

1.1.3 Biofilm inhibitors

Based on the knowledge about the processes, the genes and the regulators involved in biofilm development, different approaches to combat biofilms have been suggested (for an overview, see **Figure 1.2**) (Beloin *et al.*, 2014). Firstly, as the interaction of the bacterial cells with the surface is a crucial step in the development of a biofilm, hampering attachment can prevent biofilm formation. This can be done with non-specific surface-attached anti-adhesive polymers (Hook *et al.*, 2012). However, based on the knowledge about the adhesion process, biofilm inhibitors that function by impeding the biogenesis of adhesins (Cegelski *et al.*, 2009; Lo *et al.*, 2014; Shamir *et al.*, 2010) or that specifically bind to the receptors that mediate surface binding (Guiton *et al.*, 2012; Totsika *et al.*, 2013) were also developed. Additionally, the matrix is an important element in biofilm development. Knowledge about specific components that make up this structure made it possible to develop strategies that destabilize the matrix, for instance, by enzymatically degrading the DNA or polysaccharides (Hymes *et al.*, 2013; Kaplan *et al.*, 2004; Okshevsky *et al.*, 2015; Pleszczyńska *et al.*, 2015). Furthermore, signaling molecules or signaling pathways important for biofilm development can be targeted. Examples are anti-biofilm molecules that inhibit the activity of diguanylate cyclases, which are responsible for the synthesis of c-di-GMP, or that increase the activity of phosphodiesterases, which reduce c-di-GMP concentrations, thereby interfering with c-di-GMP signaling. Other biofilm inhibitors interfere with quorum sensing by blocking the receptors with non-natural quorum sensing molecules (reviewed in (Landini *et al.*, 2010)). These examples illustrate that understanding the cellular processes involved in adhesion, matrix production and signaling enable the rational design or identification of target-based biofilm inhibitors. Further improving the insight into the processes controlling bacterial biofilm development provides opportunities for the identification of other interesting targets for novel anti-biofilm components.

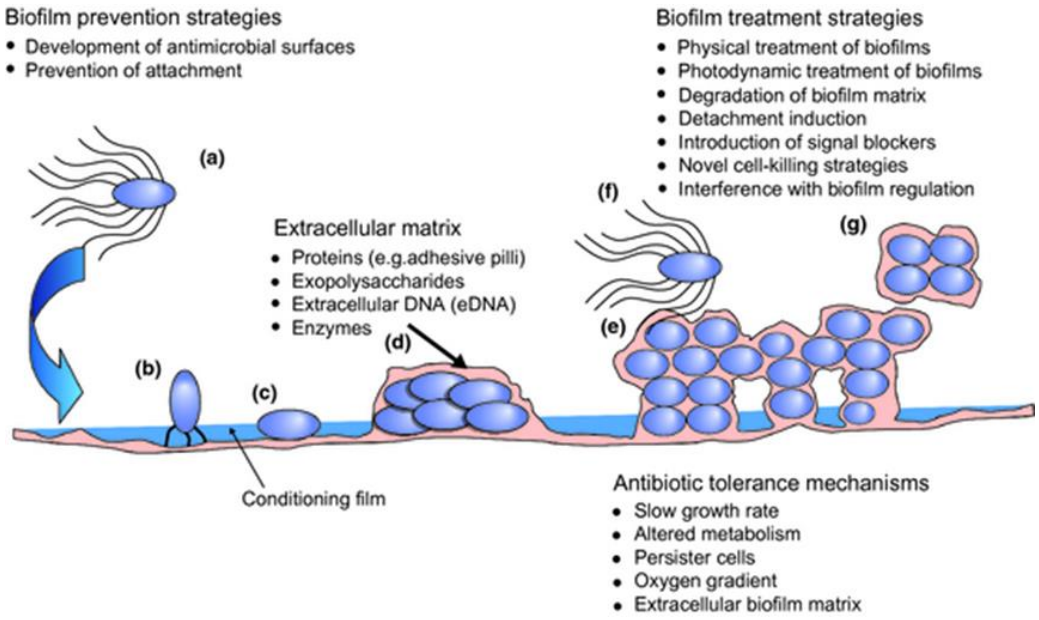


Figure 1.2 The different stages of biofilm formation include **(a)** the free-living cell, **(b)** reversible attachment to the surface, **(c)** irreversible attachment to the surface, **(d)** formation of microcolonies through cell division and extracellular matrix production and **(e)** formation of a mature three-dimensional biofilm architecture. Cells can **(f)** actively disintegrate from the biofilm or **(g)** passively be shed through mechanical disruption. Antibiotic tolerance mechanisms and anti-biofilm strategies to prevent or treat biofilms are indicated as well (Römling & Balsalobre, 2012).

1.2. Objectives and outline of the thesis

Biofilms have an increased tolerance against antibiotics, disinfectant and components of the immune system, causing problems to remove them. Therefore, there is a need for molecules that prevent biofilm formation. The increasing knowledge about the different processes and regulators involved in biofilm development already made it possible to identify small molecules that specifically target enzymes or receptors essential for biofilm formation and thereby inhibit biofilm development, as described higher. We speculate that short nucleic acid sequences and their chemically modified analogs can interfere with the expression or the activity of post-transcriptional regulators that are shown to be involved in biofilm development, providing a novel class of biofilm inhibitors.

Our hypothesis that nucleic acid sequences targeting post-transcriptional regulators of biofilm development can be interesting biofilm inhibitors, will be tested on the regulators of the CsrA-based

regulatory network in *E. coli*. CsrA, the central protein in this network, is a global regulatory RNA-binding protein, enlarging the effect of interfering with its expression or activity. Moreover, CsrA selectively binds to specific RNA sequences, which has been studied extensively, facilitating the design of decoys that bind the protein and inhibit its activity. Additionally, next to CsrA, other post-transcriptional regulators are involved in the CsrA-based network, as the activity of this protein is regulated with sRNAs, such as CsrB, CsrC and McaS. Therefore, RNA molecules that base pair to the sRNAs in this network, and thereby inhibit their function, can be tested as well. *E. coli* was chosen as a model organism because most information about the post-transcriptional regulators involved in biofilm development is available for this bacterium.

In **Chapter 1**, the process of biofilm formation was introduced together with the listing of the most important regulators of biofilm development and the biofilm inhibitors developed based on the knowledge about the process and genes involved. In this context, the role of CsrA in biofilm development was also discussed. In **Chapter 2**, the different working mechanisms of this regulatory protein are described in detail. This chapter first gives an overview of the different working mechanisms of RNA-binding proteins involved in post-transcriptional regulation. Subsequently, different RNA-binding proteins from different bacteria are described. This allows situating CsrA within the class of post-transcriptionally active RNA-binding proteins.

Chapters 3-7 describe the experimental work. In general, the experiments will reveal whether plasmid-expressed RNA sequences can affect biofilm development, whether the post-transcriptional regulators in the CsrA-based regulatory network are interesting targets for RNA-dependent inhibition and whether PNAs can be used as biofilm inhibitors that mimic the effect of the plasmid-expressed sequences. Additionally, a specific sRNA-mRNA interaction that is predicted to regulate sRNA stability will be validated.

Specifically in **Chapter 3**, it is tested whether different plasmid-expressed nucleic acid sequences, designed to interfere with CsrA or CsrB activity, affect biofilm development and growth. One of the sequences, the sense stem loop stem sequence, is further studied in **Chapter 4**, where the predicted working mechanism, i.e. targeting CsrA and consequently disturbing the expression of the genes that are regulated by this central regulatory protein, is validated. Additionally, the species-specificity of the sequence is tested. In **Chapter 5**, it is examined whether a synthetic cell-penetrating coupled PNA that is added to the growth medium, causes the same phenotypic and molecular effect as the plasmid-expressed sense stem loop stem sequence on which the sequence of the PNA is based. In **Chapter 6**, the focus is on the mirror stem loop stem sequence, another sequence that was,

unexpectedly, identified in **Chapter 3** to have an effect on biofilm development. To unravel the mechanism of action of this sequence, the genes that are differentially expressed when the sequence is transcribed, were identified, revealing which cellular processes are affected by the sequence. Additionally, possible targets of this mirror stem loop stem sequence are indicated. Finally, in **Chapter 7**, a predicted sRNA-mRNA interaction between the sRNA MicA and the *metC* mRNA, is studied. This sRNA is not involved in the CsrA-based regulatory network, but is interesting due to the nature of the predicted interaction as it is assumed to affect MicA stability. This rather unusual mechanism is further explored in this chapter. Natural regulatory mechanisms affecting sRNA stability can be an interesting source of inspiration for future design of nucleic acid based anti-biofilm molecules that target sRNA regulators of biofilm development.

Finally, **Chapter 8** lists the general discussion and overall conclusions of this work.

CHAPTER 2

LITERATURE REVIEW

RNA-binding proteins involved in post-transcriptional regulation in bacteria

Post-transcriptional regulation is a very important mechanism to control gene expression in changing environments. In the past decade, quite some interest has been directed towards the role of sRNAs in bacterial post-transcriptional regulation. However, sRNAs are not the only molecules controlling gene expression at this level, RNA-binding proteins play an important role as well. CsrA and Hfq are the two best studied bacterial proteins of this type, but recently, additional proteins involved in post-transcriptional control have been identified. This chapter focuses on the general working mechanisms of post-transcriptionally active RNA-binding proteins, which include (i) adaptation of the susceptibility of mRNAs and sRNAs to RNases, (ii) modulating the accessibility of the ribosome binding site of mRNAs, (iii) recruiting and assisting in the interaction of mRNAs with other molecules and (iv) regulating transcription terminator / antiterminator formation, and gives an overview of both the well-studied and the newly identified proteins that are involved in post-transcriptional regulatory processes. Additionally, the post-transcriptional mechanisms by which the expression or the activity of these proteins is regulated, are described. For many of the newly identified proteins, however, mechanistic questions remain. Most likely, more post-transcriptionally active proteins will be identified in the future.

This chapter was published as:

Van Assche, E., Van Puyvelde, S., Vanderleyden, J. and Steenackers, H.P. (2015). RNA-binding proteins involved in post-transcriptional regulation in bacteria. *Frontiers in Microbiology*, ***6***, 141. Doi:10.3389/fmicb.2015.00141.

2.1. Introduction

Bacteria need to survive in constantly changing environments. Therefore, they must be able to alter their gene expression in response to environmental signals, causing protein levels to be adjusted according to the needs of the cell. This can be achieved by adjusting transcription initiation with sigma factors and proteins that activate or repress transcription. However, gene expression regulation also occurs after transcription is initiated (Perez-Rueda & Martinez-Nuñez, 2012). The importance of these post-transcriptional regulatory processes is highlighted by the weak correlation that has been observed between RNA and protein abundance (Picard *et al.*, 2009).

Prokaryotic post-transcriptional regulators typically modulate RNA decay, translation initiation efficiency or transcript elongation. Different types of prokaryotic post-transcriptional regulators have been identified, including sRNAs and RNA-binding proteins. sRNAs are typically defined as non-coding RNA molecules that bind with limited complementarity near the ribosome binding site (RBS) of their target mRNA, causing competition with the ribosome for binding to this region. However, the number of sRNAs that deviate from this general definition is increasing (Liu & Camilli, 2010; Storz *et al.*, 2011). The new insights into the post-transcriptional mechanisms of sRNAs and their role in gene expression regulation were reviewed recently (Desnoyers *et al.*, 2013). Here, RNA-binding proteins involved in post-transcriptional regulation are discussed. For some of these proteins, the mechanism of action and the targets are well-described, as for CsrA and Hfq. Their post-transcriptional function in *E. coli* was already reported almost 20 years ago (Liu *et al.*, 1995; Muffler *et al.*, 1996). Lately, more insight has been gained into the diverse mechanisms these two well-studied proteins use to regulate the expression of their target genes and how they regulate their own expression or activity in *E. coli* and in other bacteria. Additional RNA-binding proteins involved in post-transcriptional regulation have been identified only recently and not much is known about their post-transcriptional function. In this review, the general working mechanisms of RNA-binding proteins are discussed first. Afterwards, examples of well-known and recently identified proteins, from *E. coli* and from other bacteria, are described.

2.2. General mechanisms of regulatory proteins that act post-transcriptionally

Bacterial post-transcriptionally active regulatory proteins typically bind RNA molecules and regulate translation initiation, stability and transcript elongation of their RNA targets, using different regulatory mechanisms. These mechanisms include (i) adaptation of the susceptibility of the target RNAs to RNases, (ii) modulation of the accessibility of the RBS of mRNA targets for ribosome binding, (iii) acting as a chaperone for the interaction of the RNA target with other effector molecules and (iv) modulation of transcription terminator / antiterminator structure formation, and will be described hereafter.

2.2.1 Adaptation of the susceptibility to RNases

Regulation of RNA stability is an important mechanism to post-transcriptionally control gene expression, as it affects the number of mRNAs that can be translated or the number of sRNAs that can execute their regulatory function. RNA stability is determined by intrinsic RNA elements, such as primary and secondary structure, but can be affected by sRNAs or proteins that bind to the RNA molecule. These proteins are mainly ribonucleases (RNases). In *E. coli*, single-stranded RNA-specific endoribonucleases (e.g. RNaseE and RNaseG) or double-stranded RNA-specific endoribonucleases (e.g. RNaseIII) generally initiate mRNA decay by making endoribonucleolytic cleavages. This yields smaller products that are further degraded by a combination of endo- and exonucleases, like PNPase (polynucleotide phosphorylase), RNaseII or occasionally RNaseR (reviewed in (Kaberdin *et al.*, 2011)). sRNAs are mainly degraded by RNaseE and PNPase, or by RNaseIII if the sRNA is hybridized to an mRNA target (reviewed in (Saramago *et al.*, 2014)).

In addition to RNases, other RNA-binding proteins can play a role in the regulation of RNA stability by modulating the susceptibility of mRNAs and sRNAs to these RNases. Regulatory RNA-binding proteins can act by directly shielding the recognition sites of RNases involved in the decay of RNA molecules if they have a shared binding preference, e.g. proteins that bind to single stranded AU-rich regions which are also recognized by RNaseE in *E. coli* (Moll *et al.*, 2003). Other regulatory RNA-binding proteins are involved in the regulation of RNA stability and induce a change in the secondary

structure of their mRNA targets upon binding. Consequently, RNase recognition sites become buried or more exposed in locally formed structures, which positively or negatively affects the RNA stability of these molecules, respectively (Barria *et al.*, 2013) (see **Figure 2.1**).

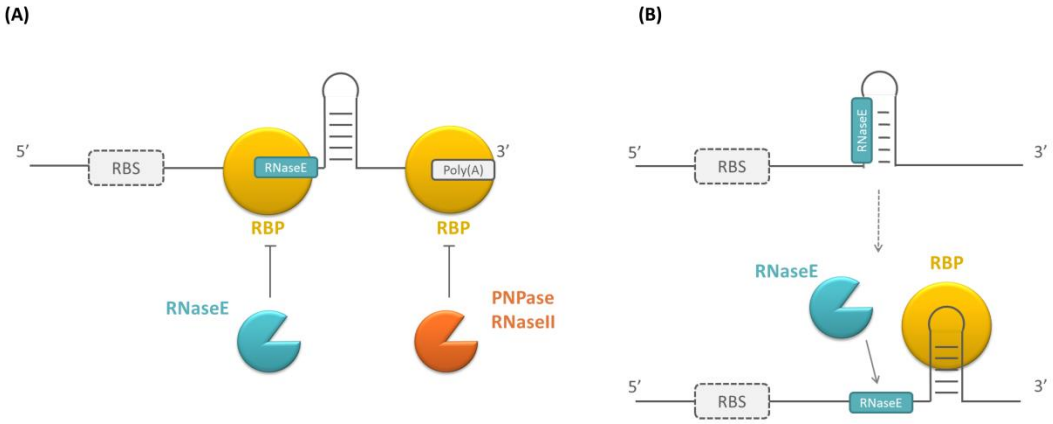


Figure 2.1 Schematic overview of the working mechanism of RNA-binding proteins influencing RNase susceptibility (A) by directly blocking RNase recognition sites or (B) by changing the secondary structure of the mRNAs they bind. Because the targets of RNA-binding proteins can be both mRNAs and sRNAs and sRNAs are not translated, the ribosome binding site (RBS) is surrounded by a dotted line. RNA-binding proteins (RBP) are depicted in yellow, endoribonucleases in blue, exoribonucleases in orange.

Although RNases are the proteins that are mainly involved in RNA degradation, mRNA modifying enzymes can facilitate mRNA turnover as well. Pyrophosphate removal at the 5’ end by RppH (pyrophosphate hydrolase) and addition of a single stranded poly(A) extension at the 3’ end of the mRNA by PAPI (Poly(A) polymerase I) both promote mRNA degradation. Additionally, the exonucleolytic decay of highly structured mRNAs can be facilitated by RhlB, which unwinds RNA structures in an ATP-dependent way (reviewed in (Kaberdin *et al.*, 2011)). *E. coli* regulatory RNA-binding proteins can interfere with the poly(A)-assisted decay of mRNA molecules by binding to the poly(A) tail and protecting the bound mRNA from degradation (Folichon, 2003). Other regulatory RNA-binding proteins facilitate mRNA degradation by recruiting RNases or RNA modifying enzymes, e.g. PAPI (De Lay *et al.*, 2013). Because these proteins form a platform for the interaction of RNA molecules and proteins, their mechanism of action is assumed to be different and will be described later.

Many components of the mRNA decay machinery, like the RNases RNaseIII, PNPase, RNaseII and RNaseR as well as the polymerase PAPI are well-conserved across the bacterial phylum (Kaberdin *et al.*, 2011). This is not the case for the major endonucleases RNaseE/G in *E. coli*. However, functional homologs of RNaseE/G were identified, e.g. RNase J1/J2 or RNaseY in *Bacillus subtilis* (Even *et al.*, 2005; Shahbabian *et al.*, 2009), which may serve as the scaffold for the degradosome in this bacterium (Hui *et al.*, 2014). Because of this conservation, it is likely that the regulatory mechanisms identified for regulatory RNA-binding proteins influencing the susceptibility to degrading or modifying enzymes in *E. coli* are conserved in other bacteria.

2.2.2 Modulating RBS accessibility

Besides their involvement in the regulation of RNA stability, RNA-binding proteins can post-transcriptionally control gene expression by altering the efficiency of translation initiation. Translation initiation of an mRNA requires ribosome binding to the RBS of the mRNA. This RBS contains the Shine-Dalgarno sequence, which is a sequence complementary to the 3' end of the 16S rRNA. This sequence is important for the recruitment and the correct positioning of the ribosome on the mRNA (Shine & Dalgarno, 1974). The more efficient the pairing between the Shine-Dalgarno region of the mRNA and the 16S rRNA, the more efficient ribosomes are recruited. Although the Shine-Dalgarno region is very important, the interaction region of an initiating ribosome is larger than the Shine-Dalgarno sequence alone and comprises nucleotides -20 to +19 relative to the start codon of mRNAs broadening the region that needs to be accessible in order for ribosome binding to occur (Beyer *et al.*, 1994; Desnoyers & Masse, 2012; Huttenhofer & Fnolier, 1994; Mackay *et al.*, 2011). Regulatory RNA-binding proteins can modulate the efficiency of translation initiation by directly competing with ribosomes for binding to the ribosome interaction region or by initiating a change in the secondary structure of the mRNA sequence near this region (see **Figure 2.2**) (Baker *et al.*, 2007; Dubey *et al.*, 2005; Irie *et al.*, 2010). The resulting reduction in translation initiation efficiency often causes mRNA stability to be decreased as well. This can be explained by two mechanisms. Firstly, RNaseE can bind internally to a transcript (Kime *et al.*, 2010; Mackie, 1998), but it can also interact with 5' monophosphorylated transcripts with its 5' binding pocket (Callaghan *et al.*, 2005). When there is no ribosome bound, the mRNA is not protected from this kind of interaction with RNaseE. Secondly, when translation initiation efficiency is reduced, the spacing of the

translating ribosomes on the mRNA is less compact. Consequently, it is more likely that RNase recognition sites in the mRNA become exposed causing transcript decay (Deana & Belasco, 2005).

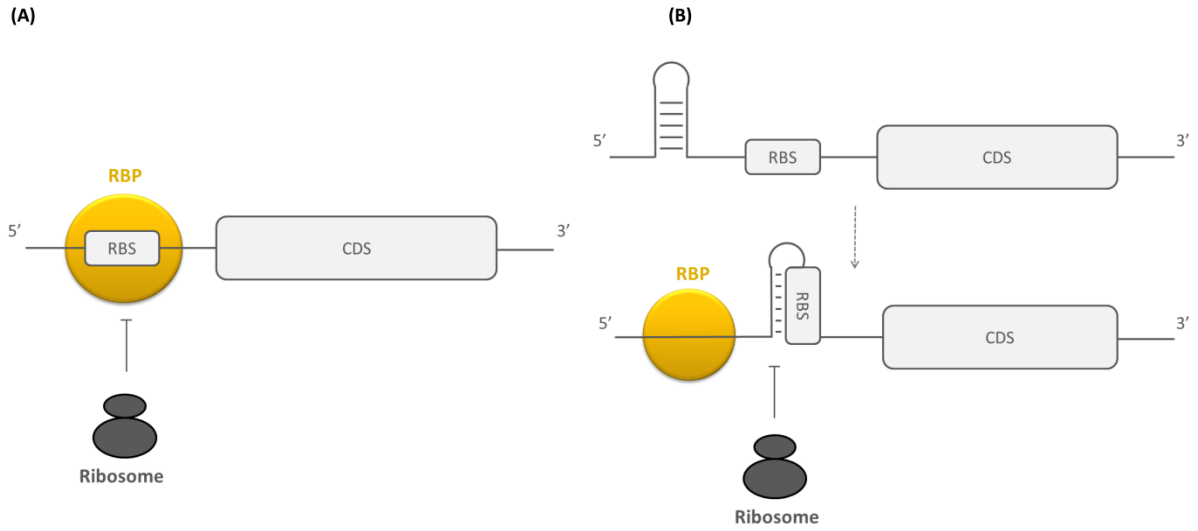


Figure 2.2 Schematic overview of the working mechanisms of RNA-binding proteins influencing the accessibility of the ribosome binding site (RBS) (A) by directly blocking this region and (B) by changing the secondary structure of the region surrounding the ribosome binding site (RBS). RNA-binding proteins (RBP) are depicted in yellow, the ribosomes in black, CDS = coding sequence.

2.2.3 Recruiting and assisting in the interaction with other molecules

RNA stability or translation initiation efficiency can also be affected by RNA-binding proteins that form a platform to assist in the interaction of other molecules, which consequently affect RNA stability or translation efficiency (see **Figure 2.3**). The mechanism of action of these RNA-binding proteins is described here as different compared to the previous two, because they typically bind simultaneously to an RNA target and an effector molecule. The effector molecule bound by the regulatory protein can be an sRNA or a protein. As previously mentioned, sRNAs typically regulate translation efficiency and RNA stability by binding near the ribosome binding site of their mRNA targets. Intermolecular base pairing between the sRNAs and mRNAs is facilitated by regulatory RNA-binding proteins that function as a chaperone (Herschlag, 1995; Soper *et al.*, 2011). The proteins recruited by regulatory RNA-binding proteins can be proteins facilitating mRNA degradation, e.g. PAPI, RNases or the degradosome (De Lay *et al.*, 2013). The degradosome is a multi-protein complex in which different components cooperate during mRNA decay. Often, it contains RNaseE as a

scaffolding protein and the protein partners PNPase, enolase and RhlB. However, its assembly is not essential for RNA decay in *E. coli* (Carpousis, 2007). The recruitment of these proteins by other RNA-binding proteins negatively affects transcript stability.

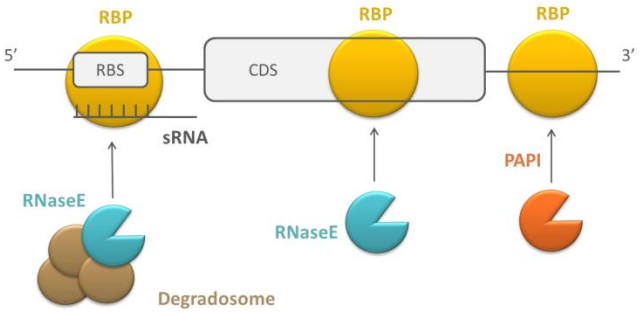


Figure 2.3 Schematic overview of the working mechanism of RNA-binding proteins that recruit and assist in the interaction of sRNA and proteins. RNA-binding proteins (RBP) are depicted in yellow, endoribonucleases in blue, exoribonucleases in orange and auxiliary factors of the degradosome in brown, CDS = coding sequence.

2.2.4 Modulation of transcription terminator / antiterminator structure formation

A last mechanism by which RNA-binding proteins can post-transcriptionally affect gene expression is by modulating transcription elongation. After RNA polymerases initiate transcription, transcripts are elongated until a terminator is reached. There are two classes of terminators: intrinsic and factor-dependent terminators. At intrinsic terminators, dissociation of the elongation complex is dependent on the nucleic acid sequence and structure, while factor-dependent termination is dependent on the action of a protein factor, like the Rho-protein (Santangelo & Artsimovitch, 2011). Typically, these terminators are present at the end of the operon. However, some also exist within the 5' leader region of the transcript. The presence of a terminator at this site prevents transcript elongation to full length. Premature termination can be abrogated by proteins that bind to the polymerase and allow transcription beyond the terminator signals or by the formation of an alternative secondary structure the enables transcription to progress (Stülke, 2002). In the latter process, RNA-binding proteins can play a role. In the case of intrinsic termination, the RNA-binding proteins can stabilize either the terminator structure or an alternative secondary structure, the antiterminator, which prevents the terminator from forming. Often the formation of both structures are mutually exclusive

(Santangelo & Artsimovitch, 2011) (see **Figure 2.4**). In general, the activity of this type of RNA-binding proteins is controlled by their phosphorylation state or by a bound ligand, which induces major conformational changes in the proteins (Santangelo & Artsimovitch, 2011), although there are exceptions (Bachem & Stülke, 1998; Tortosa *et al.*, 1997). Furthermore, RNA-binding proteins can play a role in rho-dependent termination by inducing a secondary structure change, exposing a rho utilization (*rut*) sequence that is normally inaccessible for the rho-factor. Protein binding enables access to this region and rho-dependent transcription termination takes place (Figueroa-Bossi *et al.*, 2014).

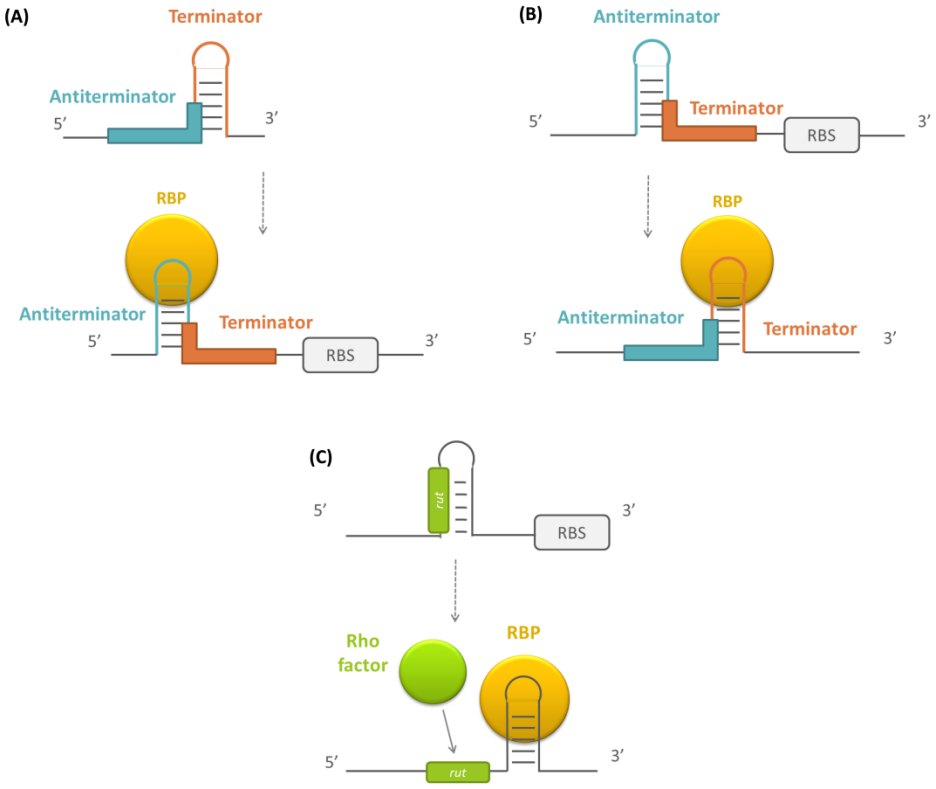


Figure 2.4 Schematic overview of the working mechanism of RNA-binding proteins that modulate transcription terminator / antiterminator structure formation (A) by stabilizing the antiterminator structure, (B) by stabilizing the terminator structure and (C) by exposing a rho utilization site. RNA-binding proteins (RBP) are depicted in yellow, the sequences forming the terminator in red, the sequences forming the antiterminator in blue and the rho utilization sequences (*rut*) en rho factor in green. RBS = ribosome binding site.

2.3. Different RNA-binding proteins that act post-transcriptionally in bacteria

A number of proteins involved in post-transcriptional regulation have been identified in *E. coli* as well as in other bacteria. They are listed in **Table 2.1**. From this list, the global regulatory proteins CsrA and Hfq are best described in literature. CsrA works predominantly by competing with the ribosome for binding to the RBS of its mRNA targets. Hfq is best known for its role in assisting interacting sRNAs and mRNAs, but the protein uses a variety of other mechanisms to post-transcriptionally control gene expression. Besides Hfq and CsrA, there are other proteins that regulate the expression of their mRNA targets using similar mechanisms. Moreover, additional RNA-binding proteins have been identified that specifically regulate sRNA stability. For other RNA-binding proteins affecting translation efficiency or RNA stability, the exact working mechanism is still unknown. Possibly they do use analogous mechanisms compared to those described for the well-known proteins.

Table 2.1. **Bacterial RNA-binding proteins that influence gene expression post-transcriptionally.** Listed in the table are the targets and processes in which the regulatory proteins are involved, the sequences they recognize, the working mechanisms they use, the molecules that regulate their activity and the bacterial species in which they were identified.

RNA-binding protein	Targets	Sequence selectivity	Predominant post-transcriptional working mechanism	Post-transcriptional regulators	Species	Reference
ANTAR containing proteins	Nitrate metabolism and other	Tandem stem-loop	Antitermination, mechanism unclear	/	Widely distributed	(Ramesh *et al.*, 2012)
Bgl/Sac family	Carbohydrate utilization genes	Ribonucleotide antiterminator (RAT)	Stabilizing antiterminator	/	Widely distributed	(Rutberg, 1997)
BpuR	*bpuR* mRNA	/	Inhibiting translation, exact mechanism unclear	/	*Borrelia burgdorferi*	(Jutras *et al.*, 2013a)
Csp	Global regulator	Hairpins	Promoting secondary structure change that changes availability for RBS or RNases Blocking RNase interactions Stabilizing antiterminator	/	Widely conserved	(Barria *et al.*, 2013)

CsrA (RsmA)	Global regulator	RUACARGGAUGU	Competing with ribosome binding Blocking RNase interactions Activation of translation by unknown mechanism	sRNAs CsrB/C (RsmX/Y/Z) (mimicry) FliW protein	Widely conserved	Reviewed in (Romeo *et al.*, 2013)
CsrD	CsrB/C sRNAs	Unspecific, specificity by accessory proteins	Unclear, possibly by promoting secondary structure change that changes availability for RNases	CsrA protein	Entero-bacteriacea	(Suzuki *et al.*, 2006)
FbpB	Iron metabolism	/	Unclear, possibly interaction platform sRNA/mRNA Possibly recruiting RNase and degradosome	/	*Bacillus subtilis*	(Gaballa *et al.*, 2008; Smaldone *et al.*, 2012)
FlbT	FliC	/	Unresolved	/	Rhizobiales *Caulobacter crescentus*	(Anderson & Gober, 2000; Ferooz *et al.*, 2011)
Hfq	Global regulator	Poly(A)/ARN Poly(U)/AU-rich ssRNA U-rich dsRNA	Interaction platform sRNA/mRNA Direct blocking of RNase recognition sites Recruiting PAPI, Crc, RNaseE and degradosome Competing with ribosome binding	Auto-regulation sRNA CrcZ	Widely conserved	Reviewed in (Sauer, 2013a)
ProQ	ProP, biofilm	Duplex with ss 5' and 3' end	Interaction platform sRNA/mRNA, ribosome association	/	*E. coli*	(Chaulk *et al.*, 2011; Sheidy & Zielke, 2013) (Chaulk *et al.*, 2011)
PyrR	Pyrimidine metabolism	/	Stabilizing anti-antiterminator		*B. subtilis*	(Lu *et al.*, 1996)
RapZ	GlmZ sRNA	/	Unclear, possibly recruiting RNaseE or promoting secondary structure change that changes availability for RNases	sRNA GlmY (mimicry)	*E. coli*	(Göpel *et al.*, 2013)
RNaseE	General RNA turnover	ss AU-rich region (A/GNAU)	RNase		Widely conserved	Reviewed in (Mackie, 2013)
RodZ	InvE (T3SS) + role in cell shape	/	Unresolved	/	*Shigella sonnei*	(Mitobe *et al.*, 2011)

RsmE	Global regulator	RUACARGGAUGU	Direct blocking of RBS	RsmA protein sRNAs RsmY/Z (mimicry)	Pseudo-monads	(Reimmann *et al.*, 2005)
RsmN/F	Global regulator	RUACARGGAUGU	Direct blocking of RBS	RsmA protein sRNAs RsmY/Z (mimicry)	Pseudo-monads	(Marden *et al.*, 2013; Morris *et al.*, 2013)
S1	Global regulator	AU-rich ssRNA	Direct blocking of RNase restriction sites	/	Gram-positive bacteria	(Hajnsdorf & Boni, 2012)
TRAP	Tryptophan metabolism	$(NAG)_{9-11}$	Direct blocking of RBS Promoting secondary structure change that blocks RBS Stabilizing terminator	Anti-TRAP protein	*Bacillus subtilis*	(Babitzke, 2004; Gollnick *et al.*, 2005; Muto *et al.*, 2000)
YbeY	Global regulator sRNAs	/	RNase Possibly interaction platform sRNA/mRNA	/	Widely conserved	(Pandey *et al.*, 2014, 2011)
YopD	T3SS + Structural pore component	/	Modifying ribosome	/	*Yersinia* species	(Chen & Anderson, 2011)

2.3.1 CsrA, an RNA-binding protein predominantly acting by changing RBS accessibility for ribosomes

CsrA of *E. coli* and its orthologs in other bacteria, are RNA-binding proteins that predominantly regulate gene expression by competing with the ribosome for binding to the RBS. CsrA is a widely conserved protein that has been annotated in over 1500 species (Finn *et al.*, 2014). The protein is a global regulator, as illustrated by the changed expression level of approximately 10 % of the genes in a *csrA* mutant in different bacteria (Brencic & Lory, 2009; Burrowes *et al.*, 2006; Lawhon *et al.*, 2003). In general, CsrA activates exponential phase functions and represses stationary phase processes (reviewed in (Romeo *et al.*, 2013)). This is caused by direct and indirect regulatory events, as some CsrA targets encode regulatory proteins themselves (Edwards *et al.*, 2011; Jonas *et al.*, 2008).

To determine the selectivity of the CsrA protein, a SELEX experiment was conducted, enabling the identification of the RNA ligands binding with the highest affinity to CsrA. The CsrA-binding

consensus sequence was shown to be RUACARGGAUGU; with the ACA and GGA being 100 % conserved. Besides by the presence of this sequence in the mRNA target, CsrA specificity is additionally determined by the secondary structure of the target, as CsrA preferentially binds to RNA molecules that have the binding motif in a hairpin structure, with GGA in the loops of the hairpin (Dubey *et al.*, 2005). The similarity between the CsrA recognition sequence and the consensus sequence of the Shine-Dalgarno region, i.e. AGGAGG, explains why there can be competition between CsrA and the ribosome for binding at this region. However, there are examples of CsrA targets that have CsrA binding sites that do not overlap with the Shine-Dalgarno region. In these cases, one of the CsrA binding sites overlaps with the translation initiation codon (Jonas *et al.*, 2008) or the binding sites are solely present within the coding region (Yakhnin *et al.*, 2011a). In the latter case, CsrA still competes with ribosome binding (Yakhnin *et al.*, 2011a). Generally, the reduced translation initiation efficiency that results from CsrA binding to an mRNA target, leads to mRNA degradation as well (Baker *et al.*, 2002; Dubey *et al.*, 2003; Wang *et al.*, 2005), although there are exceptions (Baker *et al.*, 2007).

While the predominant regulatory mechanism of CsrA is direct competition with the ribosome for RBS binding, the protein can also use other mechanisms. One example is the binding of RsmA, the CsrA ortholog in *Pseudomonas aeruginosa*, to one of its targets, *psl*, which causes a stabilization of a hairpin structure in the region spanning the RBS, blocking the Shine-Dalgarno region and preventing ribosome binding (Irie *et al.*, 2010). Secondly, binding of CsrA can have a positive effect on gene expression by blocking RNaseE interaction sites at the 5' region of the mRNA of *flhDC* in *E. coli* which has a positive effect on RNA stability (Yakhnin *et al.*, 2014). Thirdly, CsrA has been implicated recently in promoting the translation of the *moaA* mRNA. CsrA binding influences the structure of the *moaA* mRNA, however, this does not affect *moaA* mRNA levels. The exact mechanism of translational activation therefore remains to be unraveled. Remarkably, the *moaA* mRNA region that contains one of the CsrA binding sites, can also form a molybdenum cofactor (MOCO) binding riboswitch. It is unclear whether MOCO and CsrA can bind simultaneously or whether MOCO prevents CsrA from binding (Patterson-Fortin *et al.*, 2013). Lastly, CsrA induces premature transcription termination of the *pgaA* mRNA in *E. coli* by unfolding a secondary structure sequestering an entry site for transcription terminator factor Rho thereby regulating transcription elongation (Figueroa-Bossi *et al.*, 2014). Although CsrA is predominantly involved in post-transcriptional regulation, the protein has recently been shown to affect transcription as well. In *Pseudomonas protegens*, RsmA represses *lipA* transcription, although the mechanism remains unclear (Zha *et al.*, 2014). Remarkably, *lipA* is

additionally regulated by RsmE, which is one of the paralogs of RsmA in this species. RsmE blocks ribosome access by binding to the RBS of *lipA*.

Different *Pseudomonas* species indeed have three non-identical copies of RsmA, the CsrA ortholog in these bacteria. Although the expression profile of RsmE is slightly different from the one of RsmA, both proteins function in a largely redundant way (Reimmann *et al.*, 2005). Another RsmA paralog is RsmN (also called RsmF). RsmN has a different structural organization of α-helices and β-sheets compared to RsmA and RsmE, but the tertiary structure is similar. This results in a conserved spatial organization of key residues within the dimeric structure, which is necessary for RNA-hairpin recognition (Marden *et al.*, 2013; Morris *et al.*, 2013). RsmA can bind to the mRNAs of its paralogs, thereby negatively influencing RsmE and RsmN protein expression (Marden *et al.*, 2013; Reimmann *et al.*, 2005). Altogether, these elements indicate that these proteins have a unique but overlapping regulatory role compared to RsmA. It has been suggested that variations in sequence, structure, RNA-binding affinities and specificities between these different paralogs facilitate tight gene-specific control at the global post-transcriptional level for Pseudomonads. The question remains why *E. coli* does not possess this wide array of CsrA paralogs (Morris *et al.*, 2013).

2.3.2 Hfq uses different mechanisms to post-transcriptionally regulate target gene expression

Hfq is another well-studied post-transcriptional regulator. The protein is widespread but not ubiquitously present throughout the bacterial kingdom (Sobrero & Valverde, 2012). In general, a knockout in *hfq* reduces the fitness of bacteria to survive in stressful environments (Christiansen *et al.*, 2004; Liu & Camilli, 2010; Tsui *et al.*, 1994; Wang *et al.*, 2014). Its role in RNA metabolism is more limited in Gram-positive bacteria compared to Gram-negative bacteria, which is illustrated by the fact that an *hfq* deletion does not have the same global effect on the transcriptome of *Bacillus*, like it has on the transcriptome of *E. coli* or *Salmonella* (Hämmerle *et al.*, 2014).

Hfq has three different sites that can bind RNA: the distal, proximal and lateral site (Sauer, 2013a). The binding preferences of these different RNA binding sites are probably not that strict, as a study performed in *Listeria monocytogenes* shows that RNA binding sites of Hfq have the potential to bind a wider variety of RNA sequences than was previously thought (Kovach *et al.*, 2014). However, in *E. coli*, the distal site of Hfq does have a preference for repetitions of ARN-triplets or poly(A) stretches

(Link *et al.*, 2009; Lorenz *et al.*, 2010; Mikulecky *et al.*, 2004). The proximal site, on the other hand, has a preference for AU-rich single stranded sequences or poly(U) stretches (Moll *et al.*, 2003; Schumacher *et al.*, 2002). Finally, the lateral site of Hfq binds U-rich sequences and double stranded elements (Sauer *et al.*, 2012), although the function of this lateral site in RNA-binding is controversial (Sauer, 2013a). Some of the Hfq binding specificities overlap with the binding preferences of certain RNases. Therefore, Hfq can influence the RNase susceptibility of an mRNA or an sRNA. The AU-rich binding preference of the proximal binding site of Hfq, for example, is similar to the sequence that is recognized by RNaseE. Therefore, Hfq and RNaseE can compete for binding to the same region, reducing RNA decay (Massé *et al.*, 2003; Mohanty *et al.*, 2004; Moll *et al.*, 2003). In the same way, Hfq plays a role in poly(A)-assisted RNA degradation. With its distal binding site, Hfq can bind to the poly(A) tail of mRNAs. Consequently, this region becomes inaccessible for exonucleases, like PNPase and RNaseII. Hfq binding at the poly(A) tail also impairs RNaseE processing. Both processes increase mRNA stability. However, binding of Hfq can also promote polyadenylation and thus promote poly(A)-assisted decay (Le Derout, 2003; Hajnsdorf & Régnier, 2000; Régnier & Hajnsdorf, 2013). Altogether, these mechanisms indicate how Hfq is important for the stability of sRNAs and mRNAs. Although Hfq is present at high levels in the cell, there is not enough Hfq to stabilize all sRNAs and mRNAs. Therefore, there is constant competition amongst sRNAs and mRNAs for Hfq binding (Vogel & Luisi, 2011).

Next to its role in regulating RNA stability, Hfq can function as a chaperone to stimulate the interaction of mRNAs and sRNAs (Geissmann & Touati, 2004; Kawamoto *et al.*, 2006; Møller *et al.*, 2002; Rasmussen *et al.*, 2005; Zhang *et al.*, 2002). Because there are different RNA-binding sites present in a hexameric Hfq molecule, it is possible that an sRNA and an mRNA are simultaneously bound to one Hfq molecule. Such binding to Hfq brings sRNAs and mRNAs in close proximity, enhancing the likelihood of interaction (Soper *et al.*, 2011). However, cobinding of sRNAs and mRNAs to Hfq is transient and insufficient for sRNA-dependent regulation (Hopkins *et al.*, 2011). Moreover, RNA restructuring is also an important function of Hfq in this process (Henderson *et al.*, 2013). The protein can change the secondary structure of the RNA molecules, making some regions in the mRNA more accessible for base pairing (Soper *et al.*, 2011).

In addition to assisting sRNA/mRNA interactions, Hfq can also form a platform for the interaction of these RNA molecules with other proteins, e.g. RNaseE. This RNA/protein complex can then further interact with the other subunits of the RNaseE-based degradosome, causing degradation of the mRNA, and often also of the sRNA, in the complex (Aiba, 2007; Morita *et al.*, 2005). The interaction

between Hfq and RNaseE is most likely a combination of direct protein interactions between RNaseE and Hfq, which occur at the RhlB recognition region of RNaseE in the canonical degradosome (Ikeda *et al.*, 2011), and indirect interactions via the RNA molecules they bind (De Lay *et al.*, 2013). Hfq can indirectly interact in this way with a number of other proteins, like the cold shock protein CspC in *E. coli* and RsmA in *P. aeruginosa*, a protein that was discussed higher (Cohen-Or *et al.*, 2010; Sorger-Domenigg *et al.*, 2007). Another protein that may be recruited by Hfq is Crc, a protein involved in catabolite repression control in *Pseudomonas fluorescens* and *P. aeruginosa*. Crc was originally identified as an RNA-binding protein, able to bind short unpaired A-rich motifs (AAnAAnAA) at or near the RBS, thereby inhibiting translation initiation (Browne *et al.*, 2010; Moreno *et al.*, 2009; Sonnleitner *et al.*, 2009). However, a recent study shows that Crc has no RNA-binding capacity and previous results on RNA-binding rely on contaminations of Crc protein samples with Hfq (Milojevic *et al.*, 2013). Hfq and Crc are now assumed to cooperate for binding to RNAs that contain an A-rich motif, because both proteins form a co-complex and are both necessary for catabolite repression. Possibly, Hfq recruits Crc or Crc can modify Hfq in such a way that it can more efficiently bind to the A-rich stretches with its distal site, preventing translation (Moreno *et al.*, 2014).

Next to its functions as a (de-)stabilizing factor for RNA molecules and as a platform for RNA/RNA and RNA/protein interactions, Hfq carries out other functions. One of these functions is competing with initiating ribosomes for access to the RBS by binding AU-rich regions close to the RBS which are acting as translational enhancers (Desnoyers & Masse, 2012; Vytvytska *et al.*, 2000). These translational enhancers facilitate the interaction of an mRNA with protein S1 near the RBS, but this interaction is impaired when Hfq is bound. S1 is a protein, weakly associated to the 30S subunit of the ribosome that facilitates the recognition of mRNAs by ribosomes at the initial step of translation (Subramanian, 1983). Sometimes, sRNAs are involved in this regulatory process. Spot42, for example, recruits Hfq at the enhancer region (Desnoyers & Masse, 2012). In another case, Hfq binding takes place without an sRNA as a recruiting molecule. Oppositely, binding of the sRNA RyhB can prevent Hfq from binding to the enhancer region (Salvail *et al.*, 2013). In addition, Hfq has a role in transcription regulation, by inhibiting the function of the Rho protein, which is involved in transcription termination (Le Derout *et al.*, 2010; Rabhi *et al.*, 2011).

While CsrA and Hfq use different mechanisms to globally regulate gene expression, there are proteins that have only been shown to use a confined number of these mechanisms or regulate a more limited number of genes. They will be described hereafter.

2.3.3 RNA-binding proteins adapting the susceptibility for RNases

In addition to its role in facilitating ribosome interactions with the mRNA, protein S1 also has a function in post-transcriptional regulation. The protein can stabilize RNA molecules by directly shielding RNase recognition sites (Hajnsdorf & Boni, 2012). Although S1 has no strict sequence specificity, it does have a higher affinity for A/U-rich mRNA sites. As RNaseE preferentially binds AU-rich single stranded regions as well, S1 can shield RNaseE recognition sites and protect mRNAs against cleavage (Komarova *et al.*, 2005). Because S1 is capable of binding to sRNAs with the same affinity as Hfq, it has been suggested that S1 can theoretically regulate sRNA stability as well. However, the biological relevance of this suggestion still needs to be proven (Koleva *et al.*, 2006; Windbichler *et al.*, 2008).

S1 directly competes with RNases for binding to specific sequences. However, other proteins affect mRNA stability by promoting a change in the secondary structure of their mRNA targets. Consequently, RNase recognition sites become more or less available for RNases. An example of proteins that use this mechanism are cold shock proteins. These proteins are induced when bacteria encounter a temperature downshift. One of the physiological effects of cold is the stabilization of secondary structures that make RNase recognition sites inaccessible, which likewise impairs RNA degradation. Binding of the cold shock protein, CspA, to these mRNAs causes, together with the induced helicases, cold-induced secondary structures in the mRNA to be melted. Adversely, cold shock proteins can also prevent RNA degradation. CspE can bind poly(A) sites and can consequently interfere with either binding of PNPase or with internal cleavage by RNaseE (Feng *et al.*, 2001). Additionally, cold shock proteins can assist in unwinding of secondary structures that sequester the RBS, which enhances translation efficiency (Barria *et al.*, 2013).

2.3.4 RNA-binding proteins that affect sRNA stability

Hfq is very well known for its role in regulating sRNA and mRNA stability. However, recently, other RNA-binding proteins that regulate the stability of specific sRNAs were identified. A first example is CsrD (Suzuki *et al.*, 2006). In *E. coli*, this protein is involved in the turnover of the sRNAs CsrB and CsrC. These sRNAs regulate the activity of CsrA, which is an RNA-binding protein that was described

earlier. Although CsrD destabilizes the sRNAs CsrB and CsrC in an RNaseE-dependent way, these sRNAs have no obvious RNaseE recognition sites and CsrD has no RNase activity itself. Therefore, it was suggested that CsrD might induce structural changes in the sRNA, making it more susceptible for RNaseE (Suzuki *et al.*, 2006). CsrD does not bind specifically to CsrB or CsrC, however, the action of CsrD seems to be specific. This indicates that there are additional factors in the cell that determine the specificity of the process (Suzuki *et al.*, 2006). A second example of an RNA-binding protein specifically regulating sRNA stability is RapZ. RapZ is a protein identified in *E. coli* that functions as an adaptor protein guiding the processing of the sRNA, GlmZ. The protein has been reported to recruit RNaseE to the sRNA. It has been hypothesized that this occurs through changing the structure of the sRNA so it can be recognized by RNaseE, or by functioning as an interaction platform by delivering the sRNA to RNaseE (Göpel *et al.*, 2013). Most likely, there are more proteins that bind sRNAs and target them for degradation, just like RapZ and CsrD.

2.3.5 RNA-binding proteins that modulate RBS accessibility

TRAP (trp RNA-binding attenuation protein), a protein involved in the regulation of tryptophan metabolism of *B. subtilis*, acts through modulating RBS accessibility for ribosomes. The protein specifically binds multiple (9-11) NAG repeats, separated by non-conserved spacers. Because of the extended recognition sequence, TRAP can regulate a small subset of genes, all involved in tryptophan metabolism. TRAP can act as a post-transcriptional regulator by directly blocking ribosome access to the RBS (Babitzke *et al.*, 1994; Du *et al.*, 1997; Sarsero *et al.*, 2000; Yakhnin *et al.*, 2004; Yang *et al.*, 1995). Although the protein binds multiple NAG repeats, it is sufficient that one repeat overlaps with the RBS to block ribosome access to this region (Babitzke *et al.*, 1994, 1995). TRAP can also promote a change in secondary structure which influences the availability of the RBS for a subset of genes (Du & Babitzke, 1998; Merino *et al.*, 1995). Additionally, the protein is involved in the regulation of transcription elongation, which will be described later.

The regulatory RNA-binding protein BpuR from *Borrelia burgdorferi* possibly acts by competing with ribosome binding as well, as the protein has been shown to bind to the 5’ region of its mRNA target, thereby blocking translation. However, the only identified RNA target of this protein thus far is its own mRNA. Most likely, BpuR is a post-transcriptional regulator of other genes in the *Borrelia*

genome (Jutras *et al.*, 2013a). BpuR can also act as a DNA binding protein, but it binds RNA with higher affinity (Jutras *et al.*, 2013b).

2.3.6 RNA-binding proteins promoting RNA/RNA and/or RNA/protein interactions

YbeY is a widely conserved protein, known to influence the maturation of rRNAs and to be involved in the quality control of 70S ribosomes (Davies *et al.*, 2011; Jacob *et al.*, 2014). Although it is an essential RNase in some bacteria like *Vibrio cholera*, the protein is not essential and has weak RNase activity in *E. coli* and *Sinorhizobium meliloti* (Vercruysse *et al.*, 2014). In these bacteria, both *in silico* and phenotypic indications are available, supporting the hypothesis that YbeY plays a role in sRNA regulation, although YbeY has not yet been shown to actually bind sRNAs *in vivo* in these species. *In silico* analyses show that YbeY displays high sequence and structural similarities to MID domains of Argonaut proteins, the central component of sRNA-mediated gene silencing in eukaryotes. Argonaut proteins bind sRNAs that function as sequence-specific guides to lead the Argonaut proteins to perfectly or partially complementary sequences (Mallory & Vaucheret, 2010). Additionally, structural models assigned a probable RNA-binding site for YbeY. Phenotypically, there are striking similarities between an *smc01113* mutant, the *ybeY* ortholog in *S. meliloti*, and an *hfq* mutant (Pandey *et al.*, 2011). Mutated YbeY indeed causes an increased sensitivity to various stresses, similarly as when Hfq is mutated. Moreover, YbeY modulates the levels of both already identified Hfq-dependent and Hfq-independent sRNAs and their targets in *E. coli* (Pandey *et al.*, 2014), which suggests that YbeY has a central role in RNA metabolism. The exact working mechanism of the YbeY protein remains unknown. Besides the suggested role in the interaction of mRNAs and sRNAs, YbeY might still have a catalytic role as an RNase as a functional equivalent of RNaseE (Vercruysse *et al.*, 2014).

While YbeY may play a central role in the general sRNA metabolism, there are some RNA-binding proteins that form a platform of interaction for a more limited number of sRNAs. One example is the FinO family of bacterial chaperones. FinO is involved in the regulation of gene expression from the F-plasmid by facilitating the interaction between the sRNA FinP and the mRNA of the F-plasmid transcription factor *traJ*. The protein facilitates sRNA-mRNA interactions by destabilizing internal hairpins in target RNAs (Arthur *et al.*, 2003) and protects them from RNaseE (Jerome *et al.*, 1999). However, contrary to Hfq, FinO does not simultaneously bind mRNA and sRNA molecules (Chaulk *et al.*, 2010). Other proteins assigned to this FinO-family include NMB1681 in *Neisseria meningitides*

and ProQ in *E. coli*. Although NMB1681 has been shown to restore phenotypes in an *E. coli finO* mutant, the role of this protein in *Neisseria* has not been unraveled (Chaulk *et al.*, 2010). ProQ has an N-terminus homologous to FinO and a C-terminus homologous to the C-terminus of Hfq. Both the N- and the C-terminus facilitate sRNA-mRNA interactions. However, the Hfq-like domain most likely assists in sRNA-mRNA interaction, while the FinO-like domain confers sequence-specific properties to the protein (Chaulk *et al.*, 2011; Sheidy & Zielke, 2013). ProQ also associates with the ribosome, which appears to be mediated by an interaction between ProQ and its targets being translated (Sheidy & Zielke, 2013).

A second example of an RNA-binding protein possibly involved in assisting a limited number of sRNA-mRNA interactions is FbpB. FbpB is a small protein involved in the regulation of iron metabolism in *B. subtilis*. The protein is suggested to function as a coregulator of the translational repressor FsrA, by targeting the sRNA FsrA to specific transcripts and increasing the effectiveness of the sRNA. The phenotype of an *fbpB* mutant can indeed be restored by an upregulation of FsrA. FbpB is possibly involved in the recruitment of the degradation machinery of *B. subtilis* as well, thereby causing degradation of the sRNA-mRNA complex (Smaldone *et al.*, 2012). Although this is still speculative, this would be consistent with the functions of Hfq in *E. coli*.

2.3.7 RNA-binding proteins that modulate transcription terminator / antiterminator formation

TRAP and cold shock proteins have been described earlier as they can induce a change in the secondary structure of their RNA target which modulates RNA stability or translation initiation efficiency. Additionally, these proteins can modulate transcription elongation by stabilizing a transcription terminator or antiterminator structure upon binding. TRAP, activated by tryptophan binding, binds to the *Bacillus trp* 5' leader transcript and occludes the formation of the antiterminator. This antiterminator is located upstream of a terminator and the formation of both structures is mutually exclusive. Thereby, TRAP binding enables the terminator to be formed and transcription is prematurely stopped (Babitzke, 2004). The cold shock proteins CspA and homologs CspC and CspE from *E. coli*, work in the opposite way. These proteins prevent the formation of transcription terminators by stabilizing an antiterminator structure (Bae *et al.*, 2000; Phadtare *et al.*, 2002).

Another example of RNA-binding proteins that stabilize an antiterminator structure upon binding is the Bgl/Sac family. These proteins are widely distributed and recognize a 23-30 nucleotide stretch called ribonucleotide antiterminator that partially overlaps with the terminator sequence (Aymerich & Steinmetz, 1992). Similarly to cold shock proteins, binding of an RNA-binding protein of the Bgl/Sac family causes this antiterminator region to fold in a stem-loop structure that occludes the formation of the terminator. The Bgl system of *E. coli* was the first mechanism described that involves protein-mediated antitermination (Mahadevan & Wright, 1987; Schnetz & Rak, 1988). Members of this family have been identified in different bacteria such as *E. coli*, *Bacillus subtilis*, *Lactococcus lactis* and *Erwinia chrysanthemi.* They control the expression of genes required for the utilization of carbohydrates (Rutberg, 1997).

Lastly, PyrR of *B. subtilis* is involved in modulating transcription terminator / antiterminator formation, although its mechanism of action is a bit different compared to the examples described above. PyrR, activated in the presence of uridine, stabilizes an anti-antiterminator structure. This structure sequesters nucleotides of the antiterminator by base pairing with sequences that lie further upstream, inducing terminator formation and preventing gene expression. Only in the absence of uridine the antiterminator can form and expression of the RNA target is possible (Lu *et al.*, 1996).

2.3.8 Post-transcriptionally active regulatory proteins with an unknown mechanism of action

Other post-transcriptionally active regulatory proteins have been identified. However, often their exact mechanism of action remains unclear. For example, YopD is a component of the type III secretion system (T3SS) of *Yersinia* species and is conserved in pathogens with a T3SS (Schiano & Lathem, 2012). It translocates virulence factors across the cell membrane of the host. Simultaneously, YopD post-transcriptionally regulates genes of the T3SS directly and specifically in complex with LcrH, a secretion chaperone (Schiano & Lathem, 2012). The specificity of YopD is thought to be based on the interaction of the protein with short AU-rich sequences, both up- and downstream of the start codon of the target genes. However, the interaction is more complex than YopD/LcrH and AU sequences alone. Other interaction partners are involved. The post-transcriptionally active complex of YopD, LcrH and other interacting components binds to the 5'UTR

of its targets and represses their translation, but the mechanism remains unclear. YopD has been suggested to facilitate degradation of these targets by directly competing with ribosome binding or promoting degradation (Chen & Anderson, 2011). However, a recent report shows that YopD affects translation by modifying the ribosome itself (Kopaskie *et al.*, 2013). It is remarkable that a protein with a structural function has a regulatory role as well.

In *Shigella sonnei*, RodZ was identified as a membrane-localized cytoskeletal protein that retains the rod-shaped morphology of the bacterium. Later, this protein was also shown to be involved in post-transcriptional regulation, as the protein has RNA-binding capacity and its expression leads to repression of InvE protein synthesis by means of a decreased stability of the mRNA. However, the working mechanism still needs to be resolved. Possibly it forms a platform where mRNAs and other putative regulatory factors coincide (Mitobe *et al.*, 2011).

FlbT has been identified both in *Caulobacter crescentus* and *Brucella melitensis*, in which it is proposed to bind to the 5'UTR of the *fliC* mRNA. However, it is not clear how it post-transcriptionally regulates gene expression. In *Caulobacter* binding of FlbT promotes degradation of its mRNA targets, whereas in *Brucella* FlbT is proposed to be an activator of gene expression (Anderson & Gober, 2000; Ferooz *et al.*, 2011).

The family of AmiR and NasR transcriptional antiterminator regulator (ANTAR) domain proteins is a last example of RNA-binding proteins acting at the post-transcriptional level with an undefined regulatory mechanism. They are widely distributed among different species and are involved in transcription antitermination. These proteins recognize an RNA motif consisting of two tandem stem loops. However the exact molecular mechanism of antitermination has not been determined (Ramesh *et al.*, 2012). For NasR of *Klebsiella oxytoca*, it has been suggested that it does not involve the formation of an antitermination structure (Chai & Stewart, 1999).

2.4. The expression of RNA-binding proteins is often regulated by post-transcriptional regulatory mechanisms

To ensure that gene expression is adjusted according to the needs of the cell, it is crucial that the expression and the activity of the regulatory RNA-binding proteins themselves are tightly controlled as well. Remarkably, the expression or activity of these proteins is often regulated itself by post-transcriptional regulatory processes. Proteins that modulate RBS accessibility, like CsrA and BpuR, have their recognition site present in the 5'UTR of their own mRNA. Protein binding to the mRNA consequently competes with ribosome binding, reducing translation initiation efficiency and the expression of the protein (Jutras *et al.*, 2013a; Yakhnin *et al.*, 2011b). Similarly, Hfq expression is autoregulated. The protein inhibits ribosome binding to its own mRNA, making it at the same time more vulnerable for cleavage by RNaseE. Two Hfq binding sites were identified upstream of the start codon, with one overlapping with the RBS. However, the other binding site is necessary for translational repression together with a hairpin structure in the coding region. These elements possibly function as stabilizing elements for RNA/protein interaction (Vecerek *et al.*, 2005).

Secondly, the activity of different RNA-binding proteins is regulated with sRNAs. These sRNAs carry multiple high affinity sequences that are specifically recognized by a regulatory RNA-binding protein. Binding of the sRNAs sequesters the protein, resulting in a lower number of proteins available for binding to its mRNA targets. The best studied examples of sRNAs that regulate protein activity by mimicking the protein binding sequence are the sRNAs CsrB and CsrC (Liu *et al.*, 1997; Weilbacher *et al.*, 2003). They bind to the global regulator CsrA, which was discussed previously. CsrB and CsrC carry 18 and 9 CsrA binding sequences, respectively. Recently, another *E. coli* sRNA, McaS, was shown to bind CsrA. This sRNA has at least two CsrA binding sites (Jørgensen *et al.*, 2013). The different sRNA molecules that regulate CsrA activity are differentially expressed in some conditions. Consequently, the activity of the RNA-binding protein can be regulated in response to different environmental conditions (Jørgensen *et al.*, 2013). Remarkably, CsrA activity is not only regulated by sRNAs. In *Salmonella* Typhimurium, for example, the *fim* mRNA can inhibit CsrA function as well. This mRNA carries the CsrA recognition sequence but the stability or the translation of *fim* mRNA is not affected by CsrA binding, excluding it from being a regulated CsrA target (Sterzenbach *et al.*, 2013).

While CsrA is the best studied example of a protein that is regulated by sRNAs, more of them are known. The Hfq protein, which was extensively discussed above, is regulated by the sRNA CrcZ in *E. coli*. The sRNA has multiple A-rich stretches to which Hfq can potentially bind with its distal RNA-

binding region. As Hfq has other RNA-binding sites it is possible that the protein can still bind and regulate other RNA molecules when CrcZ is bound to this distal site. However, this remains to be resolved (Sonnleitner & Bläsi, 2014). A last example is RapZ, which is sequestered by binding to the sRNA GlmY. This sRNA resembles the mRNA target of RapZ, *i.e.* GlmZ, by a conserved central stem loop structure (Göpel *et al.*, 2013). This shows that not only the global regulators, like CsrA and Hfq, are regulated through sRNA mimicry.

Although some RNA-binding proteins are regulated by sRNAs, others are still regulated by proteins. This is the case for the TRAP protein, which is regulated by an anti-TRAP protein that binds near the RNA-binding pocket of TRAP, preventing it from binding to its mRNA targets (Snyder *et al.*, 2004). CsrA from *B. subtilis* is another RNA-binding protein that is regulated by a protein, FliW. This antagonistic protein binds near the active site of the protein. Remarkably, a CsrB-like sRNA has also been identified in *B. subtilis* (Mukherjee *et al.*, 2011). Its regulatory role and the importance compared to FliW has not been unraveled yet (Kulkarni *et al.*, 2006).

2.5. More RNA-binding proteins to be identified

A number of post-transcriptionally active RNA-binding proteins have been identified. However, it is very likely that more RNA-binding proteins are active as post-transcriptional regulators, given that some known regulatory RNA-binding proteins have another function, e.g. as a transcription regulator or a structural protein. Moreover, proteins that specifically regulate sRNA stability have only recently been discovered. Therefore, most likely, more proteins of this class will be identified in the future. Additionally, relatively little is known about this type of regulatory proteins in other bacteria than *E. coli*. Therefore, methods have been developed to identify new RNA-binding proteins. One of these methods is the *in vitro* or *in vivo* assembly of RNA and RNA-binding proteins, followed by mass spectrometry (Tsai et al., 2011). This method has already been optimized for *Helicobacter pylori*, *E. coli*, *Salmonella* Typhimurium and *Pseudomonas aeruginosa* (Osborne *et al.*, 2014; Rieder *et al.*, 2012; Said *et al.*, 2009; Windbichler *et al.*, 2008).

2.6. Towards applications

Additionally, further studies on well-known and newly identified post-transcriptionally active proteins will lead to a better understanding of how bacteria use this type of gene regulation to respond to changes in the environment and how different post-transcriptional networks interact with transcriptional regulons and with each other. This knowledge will generate opportunities for new or improved biotechnological applications, e.g. in synthetic biology as a tool to control gene expression, complementing the current approaches of transcription control. Additionally, as many of these proteins play a central role in RNA metabolism, interfering with the expression or the function of these proteins can be interesting as an alternative antimicrobial strategy. This application was explored in this PhD thesis and will be described in detail in the next chapters.

CHAPTER 3

INTERFERENCE WITH THE CsrA-BASED REGULATORY NETWORK

Strategies to interfere with CsrA activity and their effect on the biofilm forming ability of *E. coli*

In this chapter, RNA sequences were designed to interfere with the bacterial CsrA-based regulatory network. Herein, CsrA is the central regulatory protein of which the activity is regulated by sRNAs such as CsrB, CsrC and McaS. Because this global regulatory network plays an important role in biofilm development, it was speculated that sequences affecting CsrA or CsrB activity could impair the formation of a mature biofilm. To test this hypothesis, different sequences, designed to interfere with CsrA or CsrB function, were expressed in the cell and tested for their ability to reduce biofilm formation. This resulted in the identification of two sequences of interest. However, some of our observations regarding the biofilm forming capacity of *E. coli* strains overexpressing certain genes of the CsrA-based regulatory network are different from what was expected based on results described in literature. Here, we show that this might be explained by the fact that some genes in the network have a condition-dependent effect on biofilm development.

3.1. Introduction

In *E. coli*, CsrA post-transcriptionally regulates the expression of genes involved in gluconeogenesis, glycogen biosynthesis and catabolism (*glgCAP*), peptide transport (*cstA*), adhesin biosynthesis and its regulation (*pgaABCD* and *nhaR*), c-di-GMP synthesis (*ycdT* and *ydeH*), sRNA-dependent regulation and stability control (*hfq*), and motility (*flhDC*) (Baker *et al.*, 2007; Jonas *et al.*, 2008; Pannuri *et al.*, 2012; Romeo *et al.*, 2013; Wang *et al.*, 2005; Wei *et al.*, 2001). Some of these processes are important for biofilm development, as described in **Chapter 1**.

Besides information about the targets of CsrA, knowledge about the binding requirements of CsrA is available as well. Systematic evolution of ligands by exponential enrichment (SELEX) identified high affinity CsrA binding sequences. Consequently, a consensus sequence, RUACARGGAUGU, could be inferred, in which the underlined bases are highly conserved and are therefore important for high affinity binding (Dubey *et al.*, 2005). Next to the primary sequence, the RNA structure influences CsrA binding as well, as the presence of GGA in the loop of a short hairpin formed by base pairing of the AC and GU increases the affinity of CsrA-RNA interaction. Since part of the highly conserved sequence is then embedded within a stem, this suggests that after initial interaction, partial hairpin melting enables additional base pair contacts with CsrA (Babitzke & Romeo, 2007). Additionally, because CsrA is active as a symmetrical homodimer with two identical RNA-binding surfaces, it can bind simultaneously at two target sites within a transcript. This also affects target binding, as interaction at a lower affinity target site can be facilitated by initial binding with a neighboring higher affinity site. The distance between the two binding sites can range from 10 nucleotides to more than 63 nucleotides, but the optimal intersite distance is 18 nucleotides (Mercante *et al.*, 2010).

One or more sequences that resemble the consensus CsrA binding sequence are present in the single stranded 5'UTR of the mRNA targets of CsrA, mentioned higher. In most identified targets, one of the CsrA binding sites overlaps with the ribosome binding site, preventing ribosome access. This reduces translation efficiency and consequently, mRNA stability is often reduced as well. The mechanisms of action of CsrA were discussed in detail in **Chapter 2, section 2.3.1**. The activity of this protein is regulated by the sRNAs CsrB, CsrC and McaS. Because these sRNAs have multiple sequences that mimic a CsrA binding site, they can regulate CsrA activity through competing for protein binding with lower affinity mRNA targets (see **Figure 3.1 A**) (Babitzke & Romeo, 2007; Jørgensen *et al.*, 2013; Weilbacher *et al.*, 2003). In CsrB, 22 possible CsrA binding sequences are present in predicted loops of short RNA hairpins (see **Figure 3.1 B**) (Liu *et al.*, 1997). This high number of interaction sites might

contribute to the high affinity of CsrA for CsrB. However, the levels of CsrA normally exceed the binding capacity of the sRNAs when grown in rich medium (Gudapaty *et al.*, 2001). Unlike most other sRNAs, CsrB is not dependent on Hfq for its stability and activity. Alternatively, CsrD is involved in the regulation of CsrB degradation.

Based on the extensive knowledge about the working mechanism, the binding preferences and the interaction of CsrA and CsrB, rationally designed RNA molecules targeting CsrB and CsrA will be designed and tested for their ability to disturb the processes controlled by these regulators, such as biofilm formation.

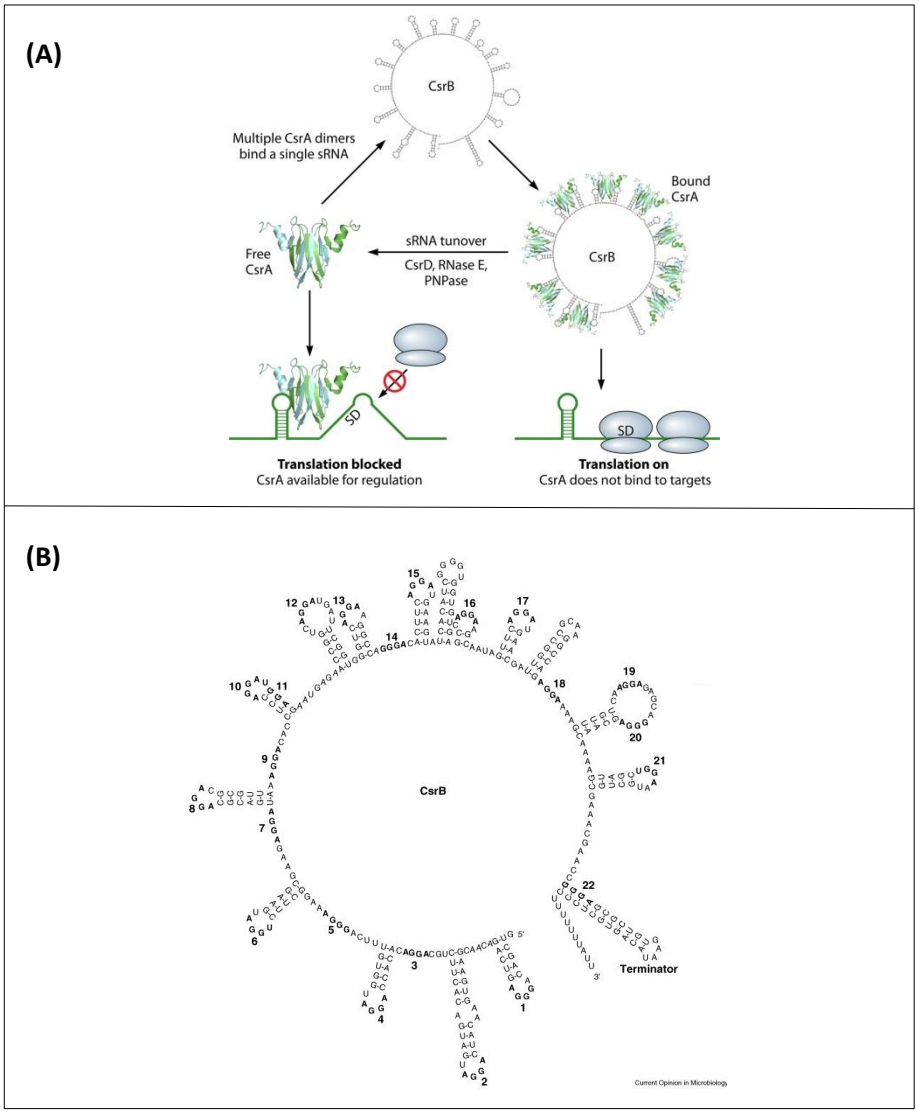


Figure 3.1 The regulatory protein CsrA. **(A) The mechanism of action of the CsrA protein and the sRNAs that regulate its activity.** CsrA is a translation regulator, generally binding to the RBS region of its target mRNAs, thereby influencing translational efficiency and mRNA stability (see **Chapter 2, section 2.3.1** for detailed information about the different working mechanisms of CsrA). The regulatory activity of CsrA is inhibited by sRNAs that sequester and antagonize CsrA proteins. CsrB and CsrC are the two best studied sRNAs with this function, but also McaS has the same activity. All of these sRNAs have several stem/loop structures resembling the regions recognized by CsrA proteins; CsrB is shown (Vakulskas *et al.*, 2015). **(B) The sequence and secondary structure of one of the CsrA sequestering sRNAs, CsrB.** Positions of the regions resembling the CsrA binding sequence are numbered (1‑22) and shown in bold. Although it is assumed that the majority of these sequences constitute authentic CsrA binding sites, some of these repeats are probably too close to one another to be recognized as discrete CsrA targets. The terminator hairpin is also shown (Babitzke & Romeo, 2007).

3.2. Materials and methods

3.2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids that were used in this chapter are listed in **Tables 3.1** and **3.2**, respectively. Bacteria were routinely grown overnight at 37°C in Luria Bertani (LB) with aeration (200 rpm). Ampicillin was added to the growth medium where needed at a concentration of 100 µg/ml.

Table 3.1 Bacterial strains

Strain name	Description	Reference
***E. coli* Top10**	F´ {*lacIq* Tn10(TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (*StrR*) *endA1 nupG*	Invitrogen
***E. coli* DH5α**	F$^-$ϕ80Δl*acZM15*Δ(l*acZYAargF*)U169 *deoP recA1endA1 hsdR17* (rk$^-$ mk$^-$)	Gibco BRL

Table 3.2 Plasmids

Plasmid name	Description	Reference
pJV853-1	Anti-*micA* expression construct based on pZE12-luc. P_{LlacO} promoter, Ap^R	Gift from J. Vogel, published in (Kint *et al.*, 2010)
pBAD/HisA	Control plasmid for L-arabinose inducible expression from an inducible pBAD promoter, Ap^R	Invitrogen
pCMPG10901	pJV853-1 based plasmid, expressing mirror stem loop stem, constructed with PRO-7172 and PRO-7336, *PstI* restriction site	This work
pCMPG10902	pJV853-1 based plasmid, expressing mirror stem loop, constructed with PRO-7172 and PRO-7316, *PstI* restriction site	This work
pCMPG10903	pJV853-1 based plasmid, expressing antisense stem loop stem, constructed with PRO-8349 and PRO-8350, *EcoRI* restriction site	This work
pCMPG10904	pJV853-1 based plasmid, expressing antisense stem loop, constructed with PRO-8349 and PRO-8351, *EcoRI* restriction site	This work
pCMPG10905	pJV853-1 based plasmid, expressing sense stem loop stem, constructed with PRO-8349 and S&P-00165, *EcoRI* restriction site	This work
pCMPG10906	pJV853-1 based plasmid, expressing sense stem loop, constructed with PRO-8349 and S&P-00166, *EcoRI* restriction site	This work
pCMPG10907	pJV853-1 based plasmid, expressing random sequence, constructed with PRO-7172 and PRO-7573, *PstI* restriction site	This work
pCMPG10908	pJV853-1 based control plasmid, no sequence incorporated, constructed with PRO-7171 and PRO-7172, *PstI* restriction site	This work
pCMPG10909	pJV853-1 based plasmid, expressing complete *csrB* sequence	This work

	(amplified with S&P-00410 and S&P-00411), incorporated in pJV853-1 amplified with PRO-7171 and PRO-8349, *EcoRI* restriction site	
pCMPG10911	pJV853-1 based plasmid, expressing *csrB* sequence in antisense, without terminator (amplified with PRO-7169 and PRO-7170), incorporated in pJV853-1 amplified with PRO-7171 and PRO-7172, *PstI* restriction site	This work
pCMPG10930	pBAD/His based plasmid expressing *ycdT* from an arabinose inducible promoter, *ycdT* was ampified with S&P00828 and S&P00829, containing *NcoI* and *XhoI* restriction sites	This work
pCMPG10931	pBAD/His based plasmid expressing *ydeH* from an arabinose inducible promoter, *ydeH* was amplified with S&P00830 and S&P00831, containing *NcoI* and *XhoI* restriction sites	This work

3.2.2 Construction of plasmids

To construct the different plasmids, standard protocols for molecular cloning were used (Sambrook and Russel, 2001). For cloning the complete *csrB* sequence in sense or antisense in pJV853-1, the *csrB* gene was amplified with PCR with one primer containing a blunt end and another with an *EcoRI* or *PstI* restriction site. The vector was amplified with PCR as well and, subsequently, vector and insert were digested and ligated.

To incorporate a specific short sequence, the template vector pJV853-1 was amplified with PCR using one primer with an overhanging end, containing the RNA sequence to be expressed and a restriction site, and one primer with the same restriction site (*PstI* or *EcoRI*). The overhanging ends were up to 20 nucleotides in length. After amplification, the PCR products were digested and religated. For the expression of *ycdT* and *ydeH* from an arabinose-inducible promoter, the YcdT and YdeH encoding genes were amplified with PCR with primers containing an *NcoI* or an *XhoI* restriction site. The pBAD/His vector was digested with the same restriction enzymes and the fragments were ligated. The primers that were used to construct the different plasmids are listed in **Table 3.3**. Ligation mixes were transferred to *E. coli* Top10 by transformation before being transferred to the desired *E. coli* strain.

Table 3.3 Primers

Primer	Sequence	Purpose	
PRO-7169	GGTTCGTTTCGCAGCATTC	Fw	pCMPG10911
PRO-7170	ATCTGCAGGTCGACAGGGAGTCAGAC	Rv	pCMPG10911
PRO-7171	GTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10908 / pCMPG10909
PRO-7172	ATCTGCAGGGCTCAGTCGAAAGACTG	Rv	Every pJV853-1 based plasmid with *PstI* restriction site
PRO-7316	ATCTGCAGATCCATGTTCCTGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10902
PRO-7336	ATCTGCAGCATGTTCCTACAGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10901
PRO-7537	ATCTGCAGCTATCTCTCTTCGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10907
PRO-8349	ATGAATTCGGCTCAGTCGAAAGACTG	Rv	Every pJV853-1 based plasmid with *EcoRI* restriction site
PRO-8350	ATGAATTCGTACAAGGATGTGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10903
PRO-8351	ATGAATTCTAGGTACAAGGAGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10904
S&P-00165	ATGAATTCACATCCTTGTACGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10905
S&P-00166	ATGAATTCTCCTTGTACCTA GTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10906
S&P-00410	ATGAATTCCTGCCGCGAAGGATAGCAG	Fw	pCMPG10909
S&P-00411	GTCGACAGGGAGTCAGACAACG	Rv	pCMPG10909
S&P-00828	ATCCATGGCAGATTGGTGTAGCTTTATGG	Fw	pCMPG10930
S&P-00829	ATCTCGAGCCGCTTTATGGTGACTCAC	Rv	pCMPG10930
S&P-00830	ATCCATGGGGAGTGGCAATGATCAAGAAG	Fw	pCMPG10931
S&P-00831	ATCTCGAGAACGGAGCTTAAACTCGG	Rv	pCMPG10931

3.2.3 RNA isolation

RNA samples of free-living cells were taken after growing the cells in a flask. At the desired OD_{595}, 20 % (v/v) of stop solution (5:95 phenol:ethanol (v/v)) was added to an equivalent of OD_{595} = 1. Biofilm samples were taken by growing the cells in a petri dish. After a specific time, the medium was poured off and the cells attached to the bottom of the petri dish were scraped off and suspended in 1 ml 1/20 TSB. Subsequently, 200 µl of stop-solution was added. In both cases, the mixture of cells and stop solution was snap-frozen in liquid N_2. RNA was isolated with the 'ToTALLY RNA Total RNA isolation kit' (Ambion; isolation through phase separation) for Northern blot analyses of the short sequences. For all other purposes the 'SV Total RNA Isolation Kit' (Promega; isolation based on the selective binding to a membrane) was used. RNA isolation was done according to the protocol of the manufacturer. DNA in the sample was removed with the Turbo DNA-free kit (Ambion) and the

samples were further purified and concentrated by isopropanol precipitation (0.1 volume NaOAc and 2.5 volume ice cold isopropanol). The concentration and the quality of the RNA sample was checked with a spectrophotometer (Nanodrop, Thermo Scientific) or via capillary gel electrophoresis (Experion, Bio-Rad).

3.2.4 Northern blot analysis

To visualize short RNA molecules on northern blots, 5 - 10 µg of total RNA was separated on a 15 % polyacrylamide / 6 M ureum gel containing 0.1 % (v/v) tetramethylethylenediamine (TEMED) and 0.1 % (m/v) ammonium persulphate (APS) in TBE-buffer (100 mM Tris; 82.5 mM Boric Acid; 1.1 mM EDTA). To the RNA samples and the ladder (RiboRuler Low Range, Fermentas), loading dye (2X; Fermentas) was added and the samples were denatured at 70 °C for 5 and 10 minutes, respectively, before being loaded on the gel. After separation, the RNA ladder was cut off and colored using ethidium bromide. The RNA samples were electrically transferred through semi-dry blotting (Electrophoresis Power Supply, Amersham Pharmacia Biotech) to a nylon membrane (Roche) and were cross-linked with UV irradiation. After pre-incubating the membrane in the hybridization buffer at 68 °C for 1 hour (OligoHyb, Ambion, 1 ml / 10 cm^2), the DIG-labeled probe (100 ng/ml) was added and left to hybridize overnight at 40 °C. The probes used are listed in **Table 3.4**. After hybridization, the membrane was washed in buffer 1 (100 mM maleic acid, 150 mM NaCl; pH 7.5) and blocked in buffer 2 (1 % (m/v) blocking reagent (Roche), dissolved in buffer 1). Hereafter, the anti-DIG antibody bound to alkaline phosphatase (Anti-Digoxigenin Fab fragments, 0.75 U / µl; Roche) was added to enable it to bind to the probe. The signal was detected by adding 10 µl CSPD (chloro-5-substituted adamantly-1,2-dioxetane phosphate) in 990 µl buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5). Unbound antibodies were washed away with buffer 1. The signal was detected on a photographic film (Fujifilm).

Table 3.4 DIG-labeled probes for northern blot detection

Probe	Sequence	Target	Reference
PRO-0157	CTACGGCGTTTCACTTCTGAGTTC	5S rRNA (loading control)	(Papenfort *et al.*, 2006)
PRO-8407	AATTCGTACAAGGATGT	Antisense stem loop stem	This work

3.2.5 cDNA synthesis and qRT-PCR analysis

cDNA was made from 500 ng of the isolated and purified RNA with the Revert Aid H minus cDNA synthesis kit (Thermo Scientific). After dilution, 5 µl of cDNA (2 ng/µl), 0.9 µl of each primer (20 µM) and 3.2 µl qRT-PCR water (Ambion) were added to 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems). The qRT-PCR reactions were performed in the StepOnePlus real time PCR cycler (Life Technologies) and were done in 3 technical repeats. The primers used for qRT-PCR analysis are listed in **Table 3.5**. The expression of *rrnD* (encoding the ribosomal operon *rrsD-ileU-alaU-rrlD-rrfD-thrV-rrfF*), *rpsS* (encoding the 30S ribosomal subunit protein S19) and *gyrA* (encoding DNA gyrase subunit A) are included as endogenous controls. The expression levels of these housekeeping genes were used to normalize the expression levels of the target genes.

Table 3.5 qRT-PCR primers

Primer	Sequence	Purpose		Reference
PRO-3380	GTCAGCACGTTCCGGTATTTG	Fw	rpsS	H. Steenackers
PRO-3381	GCGAATTCACCCAGTTTGTGA	Rv	rpsS	H. Steenackers
PRO-4253	CGCACGGCCAACAATGA	Fw	gyrA	K. Hermans
PRO-4254	CAACATTGAGGAGGAGCTGAAGA	Rv	gyrA	K. Hermans
PRO-7841	AGTTCCAGTGTGGCTGGTCAT	Fw	rrnD	(Jonas et al., 2008)
PRO-7842	GCTCACCAAGGCGACGAT	Rv	rrnD	(Jonas et al., 2008)
PRO-7847	TCGAACGTGAACCGCAAGA	Fw	pgaA	(Jonas et al., 2008)
PRO-7848	ATGTACATCAACCGCACGTTTT	Rv	pgaA	(Jonas et al., 2008)
S&P-00496	GAAGAAGGCATCGTGCTGGTA	Fw	glgC	This work
S&P-00497	TCGCTCCTGTTTATGCCCTAA	Rv	glgC	This work
S&P-00502	ACGCCTTATTGCGTCATGATT	Fw	ycdT	(Jonas et al., 2008)
S&P-00503	CCCCAGGTGTCGTTGACTTT	Rv	ycdT	(Jonas et al., 2008)
S&P-00504	AATAAGGCTATCGATGCCCACTAC	Fw	ydeH	(Jonas et al., 2008)
S&P-00505	CGCGACCACGCTGTGA	Rv	ydeH	(Jonas et al., 2008)
S&P-00858	ACACCAATAACAGCCAGCTCTTC	Fw	fliA	This work
S&P-00859	TGCTATCGCCGTGCTCTTC	Rv	fliA	This work

3.2.6 Biofilm assay

Biofilm formation was tested in 1/20 TSB at 25°C, unless stated otherwise. To test the biofilm forming capacity of different strains, bacterial cultures, grown overnight as described in **section 3.2.1**, were diluted 1/100 in 1/20 TSB, with a correction to OD_{595} of 2.5. Where appropriate, antibiotics and isopropyl-β-D-1-thiogalactopyranoside (IPTG; 1 mM) were added and these cultures were grown in non-shaking conditions for 24 or 48 hours in the wells of the Calgary Biofilm Device (De Keersmaecker *et al.*, 2005). This device consists of a lid of 96 pegs which fits perfectly on a 96-well microtiter plate (Nunc – Thermo Scientific). This enables an easy transfer of the bacterial cells that have attached to the pegs during incubation. When biofilms were grown for 48 hours, the medium was refreshed after 24 hours, transferring the lid to a microtiter plate containing fresh medium, antibiotics and IPTG, if necessary. After the desired incubation time, pegs were washed in 200 µl PBS and stained for 30 minutes with 200 µl crystal violet per well (0.1 % crystal violet in 1:1:18 isopropanol:methanol:PBS (v/v)). After washing off the excess crystal violet in distilled water, the pegs were left to dry on the air for 30 minutes. Afterwards, the pegs were destained in 30 % acetic acid (200 µl/well). The OD_{570} of this destain, corresponding to the amount of crystal violet taken up by the cells and the matrix and thus representing the amount of biofilm formed on the pegs, was subsequently determined. Optical densities were measured in a microplate reader (Synergy MX microtiter plate reader, Biotek Instruments, Inc.). For accurate comparison of data between different plates, a strain with an empty plasmid or with a plasmid expressing a random sequence was included in every plate. This random sequence has the same length and GC content as the short sequences and was generated using the Random DNA sequence generator (http://www.faculty.ucr.edu/~mmaduro/random.htm). For data analysis, the amount of biofilm formed was compared to the amount of biofilm formed by the strain with an empty vector or expressing a random sequence. The results are representative for three biological repeats, in which every strain was tested in six technical repeats, unless stated otherwise in the text.

3.2.7 Growth assay

Growth assays were performed in 1/20 TSB at 25°C, unless stated otherwise. To compare the growth of different strains, cells were diluted 1/5000 from an overnight culture, grown as described in **section 3.2.1**. These diluted cultures were grown in a 'honey comb' plate (Thermo Scientific) under shaking conditions, at the appropriate temperature and with the addition of antibiotics and IPTG (1 mM), where necessary. The OD_{600}, representing the amount of cells present in the medium, was measured every 15 minutes (BioscreenC, Labsystems Oy). The results are based on four technical repeats.

3.3. Results

3.3.1 Design of the sequences

Different RNA-based strategies were developed to interfere with CsrA activity, thereby aiming at disturbing the expression of the genes that are regulated and the processes that are controlled by CsrA, specifically biofilm formation. The first strategy is based on expressing the reverse complementary sequence (**antisense**) of the regulatory sRNA CsrB. Base pairing between the reverse complementary *csrB* sequence and CsrB itself is predicted to prevent the interaction between CsrB and CsrA, thereby increasing the level of CsrA available for binding other targets. Although multiple sRNAs regulate CsrA activity in *E. coli*, a *csrB* mutant has been shown to have a reduced biofilm forming capacity (Jackson *et al.*, 2002) and therefore we expect that interfering with the activity of only this sRNA is sufficient to affect biofilm development. The second strategy is based on overexpressing *csrB* (**sense**). This is predicted to increase CsrA sequestration, which reduces CsrA availability and impairs its regulatory activity. For expressing the *csrB* sequence in sense, the complete sequence was amplified from start to stop codon. For expressing the *csrB* sequence in antisense, the terminator loop of *csrB* was excluded. This was done to prevent premature transcription termination of the complete antisense sequence. Indeed, if this region was included, there would be a stable terminator loop followed by a U-rich sequence, which are signals for rho-independent termination.

Expressing the complete *csrB* sequence in sense or antisense is assumed to be the most efficient way to disturb the regulatory CsrA-based network and affect biofilm development. However, it would be more interesting to find short RNA sequences with the same activity, as shorter sequences can be taken up more easily by the cells when they are exogenously added to the medium. This is necessary if they are used as biofilm inhibitors. Therefore, next to the complete *csrB* sequence, different short sequences that are complementary or identical to the regions within CsrB that interact with CsrA were tested, as these regions are crucial for CsrB action (Babitzke & Romeo, 2007). The exact sequences that are used throughout this chapter are listed in **Table 3.6**.

Table 3.6 Sequences encoded on the different plasmids. The SELEX-derived consensus CsrA binding site is given as well, as regions within CsrB that mimic this sequence are important for CsrB activity. The GGA, essential for CsrA binding, is indicated in bold.

Description	Sequence
SELEX-derived consensus CsrA binding site	RUACAR**GGA**UGU
Antisense stem loop stem	ACAUCCUUGUAC - RS[1] - terminator
Antisense stem loop	UCCUUGUACCUA - RS[1] - terminator
Sense stem loop stem	GUACAA**GGA**UGU - RS[1] - terminator
Sense stem loop	UAGGUACAA**GGA** - RS[1] - terminator
Mirror stem loop stem	UGUA**GGA**ACAUG - RS[1] - terminator
Mirror stem loop	A**GGA**ACAUCCAU - RS[1] - terminator

[1] either *PstI* or *EcoRI* restriction site

The **antisense stem loop stem (Antisense SLS)** sequence is the reverse complement of the regions within CsrB interacting with CsrA, which resemble the consensus CsrA binding site. However, this sequence can form a stem loop structure, which might prevent efficient binding to CsrB. Therefore, another construct was additionally made, encoding a sequence that can only base pair to one side of the stem and the loop of the consensus sequence, called the **antisense stem loop (Antisense SL)**. This sequence is complementary to part of the CsrA binding region, including the GGA that is typically present in every identified CsrA binding site, which should be enough to reduce CsrB action because of sterical hindrance. Interaction of this sequence with CsrB might be more easy as this short sequence is less structured. The **sense stem loop stem (Sense SLS)** sequence is a decoy and exactly mimics the CsrB region interacting with CsrA, which resembles the consensus CsrA binding sequence. The **sense stem loop (Sense SL)** sequence encodes only part of the consensus binding site, including

the typical GGA. Similar to the constructs encoding the short antisense or sense sequences, constructs were made that express the short sense sequences in reverse orientation **(Mirror SLS and Mirror SL)**. However no effect on CsrA function is expected for these sequences. A schematic representation of the sense, antisense and mirror sequences is depicted in **Figure 3.2**.

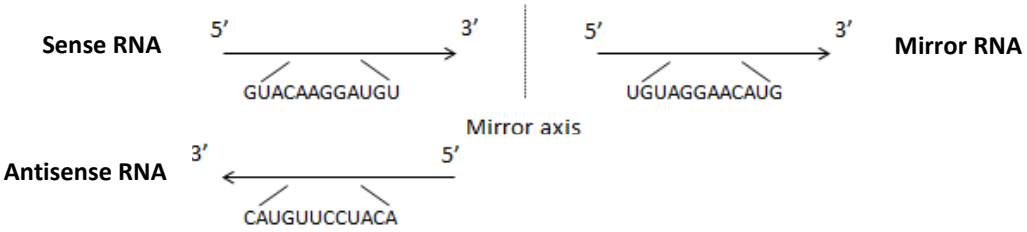


Figure 3.2 Schematic representation of the designed sense, antisense and mirror sequences. The stem loop stem (SLS) sequence is given.

3.3.2 Expression analysis

The sequences listed in **Table 3.6** are encoded on a plasmid, which is based on pJV853-1. In this vector, transcription starts exactly at the beginning of the desired sequence and no RBS is present between the promoter and the start of the RNA sequence. Additionally a terminator structure is encoded on the plasmid. This plasmid was used before to express the sequence of the sRNA MicA in antisense to deplete its expression (Kint *et al.*, 2010). The expression of the short sequences from this plasmid was tested using northern blot analysis (see **Figure 3.3**). Hereto, RNA samples were taken from *E. coli* DH5α cells with the antisense stem loop stem plasmid (pCMPG10903) and cells with a plasmid encoding a random sequence with the same length and the same GC content as the antisense stem loop stem sequence (pCMPG10907) grown for 24 or 48 hours in 1/20 TSB at 25°C without shaking. These conditions were chosen because of their practical relevance, as a representation for biofilms grown under ambient and low-nutrient conditions, like on industrial surfaces. The expression of these sequences was induced by adding IPTG to the growth medium. Samples were taken from the biofilm cells which are attached to the surface of the petri dish. The specific antisense RNA sequence was detected using a DIG-labeled probe (PRO-8407). Detection with the 5S rRNA probe was included as loading control (PRO-0157). The results are shown in **Figure 3.3**.

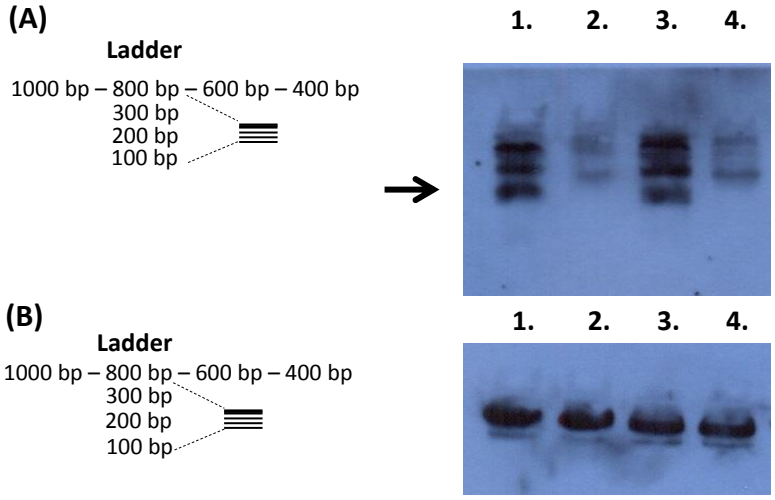


Figure 3.3 Northern blot analysis of (A) the antisense stem loop stem sequence using PRO-8407 and (B) 5S rRNA using PRO-0157. RNA samples were taken from the biofilm fraction, from cells grown non-shaking in 1/20 TSB at 25°C from cells with a plasmid encoding **1.** antisense stem loop stem sequence (pCMPG10903) grown for 24 hours **2.** random sequence (pCMPG 10907) grown for 24 hours **3.** antisense stem loop stem sequence (pCMPG10903) grown for 48 hours **4.** random sequence (pCMPG 10907) grown for 48 hours. The separation of the RNA ladder is based on ethidium bromide coloring before transfer to the membrane and therefore, the position of the length markers relative to the blot is an approximation. Exposure times are not equal between panel (A) and (B).

In **Figure 3.3**, a specific band, indicated with an arrow, was observed in the RNA samples from cells containing a plasmid that encodes the antisense stem loop stem sequence (pCMPG10903) in comparison to the samples from cells containing the plasmid that encodes a random sequence (pCMPG10907). This confirms that the cells express the antisense stem loop stem RNA sequence under biofilm conditions. However, some non-specific bands could be detected as well.

3.3.3 Effect on biofilm development and growth

Subsequently, *E. coli* DH5α strains with plasmids expressing the different sequences listed in **Table 3.6**, were tested for their biofilm forming capacity. Firstly, the strategy based on the expression of reverse complementary sequences was evaluated. Hereto, both the complete reverse complementary *csrB* sequence (Antisense CsrB - pCMPG10911) and two sequences that are the reverse complement of different parts of the regions within CsrB responsible for interacting with CsrA, resembling the consensus CsrA binding sequence (Antisense SLS - pCMPG10903 and Antisense SL - pCMPG10904), were expressed in *E. coli* DH5α. The biofilm forming capacity of these strains was compared to that of bacteria having a control plasmid (the empty plasmid - pCMPG10908 and a plasmid containing a random 12 nt sequence that has the same GC content as the short antisense sequences - pCMPG10907). Also the growth of the strains expressing these sequences was measured. The results are shown in **Figure 3.4** and **3.5**. These figures show that neither expressing the complete *csrB* sequence in antisense (pCMPG109011), nor expressing the short sequences based on the CsrA binding sites within CsrB in antisense (pCMPG10903 and pCMPG10904), significantly affect the biofilm forming ability or the growth of the strains.

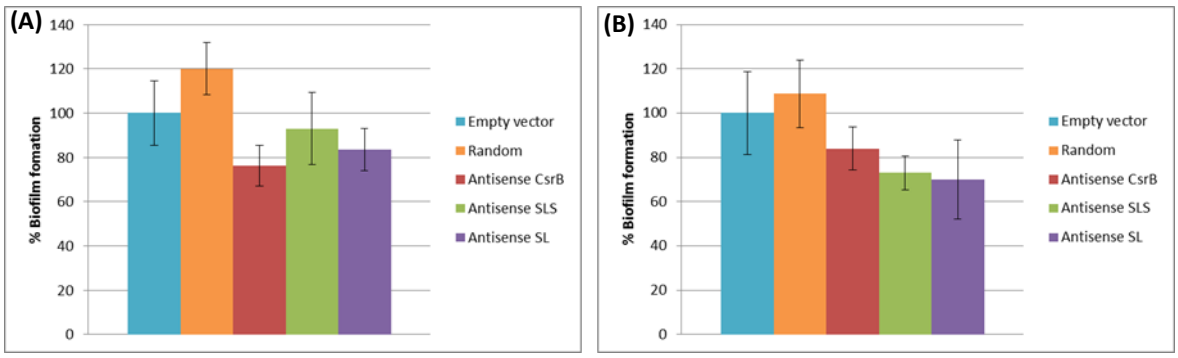


Figure 3.4 Biofilms formed by *E. coli* DH5α strains with plasmids expressing *csrB* or the regions within CsrB interacting with CsrA in antisense (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), antisense CsrB (pCMPG10911), short antisense stem loop stem (Antisense SLS - pCMPG10903), short antisense stem loop (Antisense SL - pCMPG10904). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

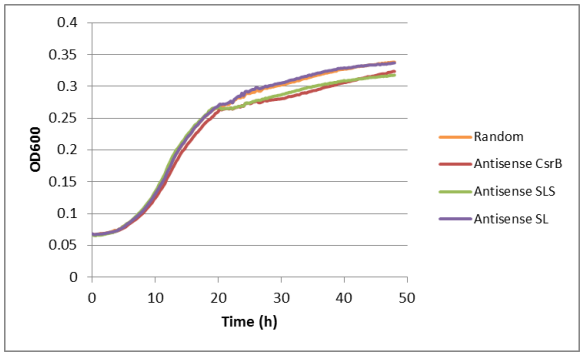


Figure 3.5 Growth of *E. coli* DH5α strains with plasmids expressing *csrB* or the regions within CsrB interacting with CsrA in antisense, continuously monitored with the Bioscreen. The results are shown for strains expressing a random sequence (pCMPG10907), antisense CsrB (pCMPG10911), short antisense stem loop stem (Antisense SLS - pCMPG10903), short antisense stem loop (Antisense SL - pCMPG10904).

Secondly, the strategy based on the overexpression of the sRNA was evaluated. Therefore, the complete *csrB* sequence (Sense CsrB - pCMPG10909) and two short sequences based on the regions within CsrB responsible for interacting with CsrA (Sense SLS - pCMPG10905 and Sense SL - pCMPG10906), resembling the consensus CsrA binding sequence, were expressed in sense direction. Strains expressing these sequences were tested for their biofilm forming capacity. The results are shown in **Figure 3.6**. Additionally, the effect of expressing these sequences on the growth of the strains was tested, as shown in **Figure 3.7**.

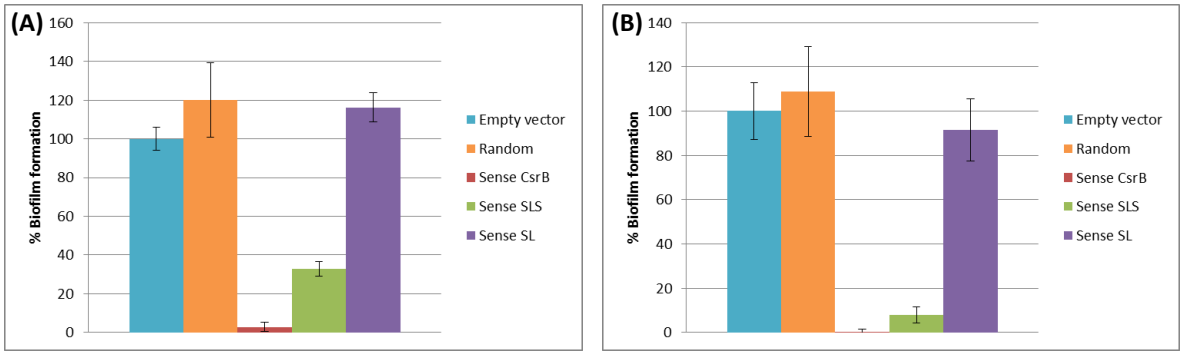


Figure 3.6 Biofilms formed by *E. coli* DH5α strains with plasmids expressing *csrB* or the regions within CsrB interacting with CsrA in sense (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), sense CsrB (pCMPG10909), short sense stem loop stem (Sense SLS - pCMPG10905), short sense stem loop (Sense SL - pCMPG10906). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

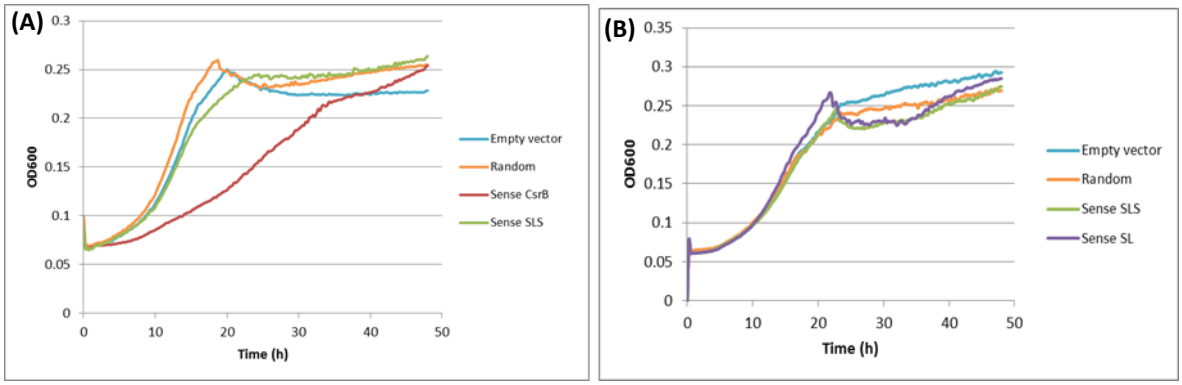


Figure 3.7 Growth of *E. coli* DH5α strains with plasmids expressing *csrB* or the regions within CsrB interacting with CsrA in sense, continuously monitored with the Bioscreen. The results are shown in **(A)** for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), sense CsrB (pCMPG10909), short sense stem loop stem (Sense SLS - pCMPG10905) and in **(B)** for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), short sense stem loop stem (Sense SLS - pCMPG10905), short sense stem loop (Sense SL - pCMPG10906).

The results in **Figure 3.6** show that the amount of biofilm formed by strains expressing the complete *csrB* sequence (pCMPG 10909) is drastically reduced compared to the amount of biofilm formed by strains with an empty vector (pCMPG 10908) or strains expressing the random sequence

(pCMPG10907). A reduction in biofilm formation was also observed for strains with one of the short sense constructs, expressing the stem loop stem sequence (pCMPG10905). Expressing the sense stem loop sequence (pCMPG10906), which only contains part of the consensus CsrA binding site, does not cause an effect on biofilm formation. The growth results presented in **Figure 3.7** show that expressing the complete *csrB* sequence in sense affects growth, but expressing the short sense stem loop stem (pCMPG10905) or the short sense stem loop sequence (pCMPG10906) has no effect on the growth pattern of the strains when compared to strains with an empty vector or strains expressing a random sequence.

Finally, strains expressing the mirror stem loop stem (Mirror SLS - pCMPG10901) and the mirror stem loop (Mirror SL - pCMPG10902) sequences were tested for their ability to form a biofilm and to grow. The results are shown in **Figure 3.8** and **3.9**.

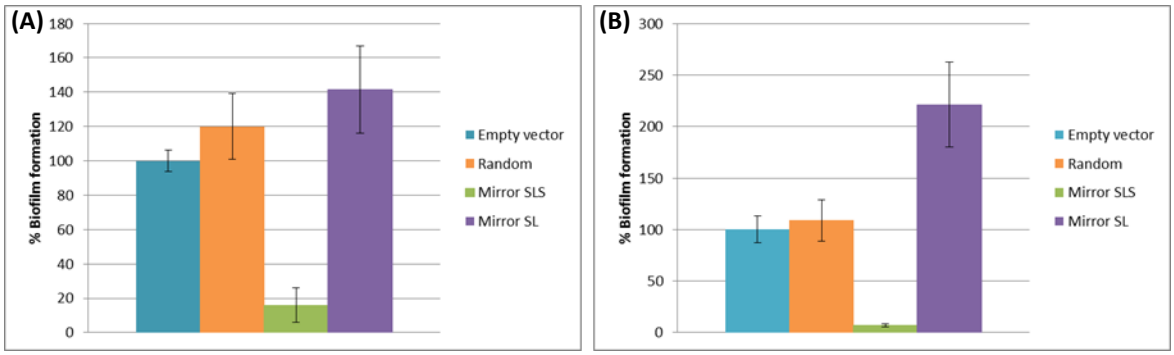


Figure 3.8 Biofilms formed by *E. coli* DH5α strains with plasmids expressing the mirror sequences (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), short mirror stem loop stem (Mirror SLS - pCMPG10901), short mirror stem loop (Mirror SL - pCMPG10902). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

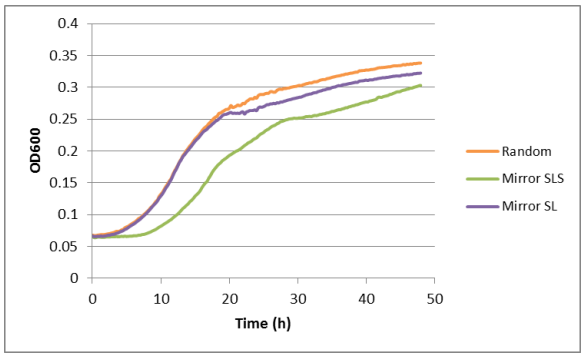


Figure 3.9 Growth of *E. coli* DH5α strains with plasmids expressing the mirror sequences, continuously monitored with the Bioscreen. The results are shown for strains expressing a random sequence (pCMPG10907), short mirror stem loop stem (Mirror SLS - pCMPG10901), short mirror stem loop (Mirror SL - pCMPG10902).

From **Figure 3.8** it is clear that expressing the mirror stem loop stem sequence (pCMPG10901) drastically reduces the biofilm forming capacity of the strain, while the mirror stem loop sequence (pCMPG10902) increases biofilm formation, but only after 48 hours of growth. The amount of biofilm that is formed by strains expressing the mirror stem loop stem sequence (pCMPG10901) is less than by strains expressing the sense stem loop stem sequence (pCMPG10905), but more than by strains expressing the complete *csrB* sequence in sense (pCMPG10909) (see **Figure 3.6**). Also, the growth of the strains expressing the mirror stem loop stem sequence is reduced (see **Figure 3.9**).

3.3.4 Condition-dependency of CsrB regulation

Our observation that strains with the *csrB* overexpression plasmid (pCMPG10909), which induces a forced and uncontrolled expression of *csrB*, have a reduced biofilm forming capacity in *E. coli* was unexpected, as it has been reported previously that overexpressing *csrB* increases the amount of biofilm that is formed by this bacterium (Jackson *et al.*, 2002). Therefore, the expression levels of a number of known CsrA targets were validated in cells with pCMPG10909, expressing the complete *csrB* sequence. As CsrB is known to sequester CsrA, preventing CsrA from binding to its targets, it is expected that the expression of the CsrA targets is no longer repressed in the presence of the *csrB* overexpression plasmid. The expression levels of the CsrA targets were measured with qRT-PCR. Although CsrA predominantly affects translation efficiency, for all CsrA targets tested here, the

mRNA stability is affected upon CsrA binding as well. As the stability of the mRNA affects the expression level of the gene, this can be detected with qRT-PCR. The results are shown in **Figure 3.10**. RNA samples were taken from exponentially growing cells (OD 0.1) in 1/20 TSB at 25°C.

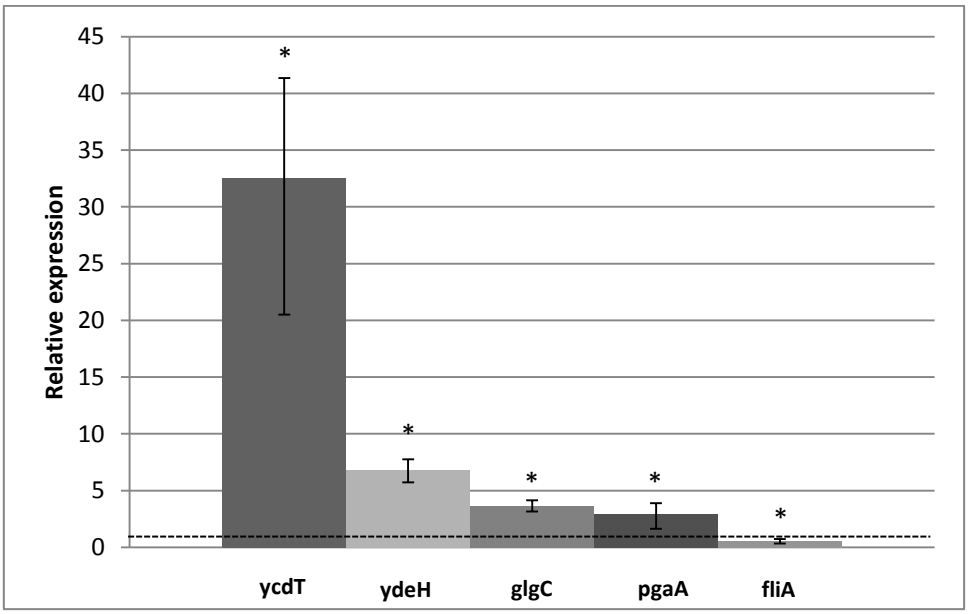


Figure 3.10 Relative expression levels of a selected number of CsrA targets in cells with the *csrB* overexpression plasmid (pCMPG10909) relative to cells with a control plasmid (pCMPG10908) for cells grown in 1/20 TSB at 25°C. Error bars represent 95% confidence intervals. A star indicates that the C_T values are significantly different for both strains ($p < 0.05$). The dotted line indicates a relative expression level of 1. Relative expression levels > 1 indicate increased expression, relative expression levels <1 indicate reduced expression compared to the control strain.

The results from the gene expression study in **Figure 3.10** show that the expression of *ycdT*, *ydeH*, *glgC* and *pgaA* is upregulated when the complete *csrB* sequence is overexpressed from pCMPG10909 when compared to cells with an empty vector. These results show that these known CsrA targets are not repressed by CsrA when *csrB* is overexpressed, consistent with the notion that increased levels of this sRNA lead to higher CsrA sequestration, rendering less CsrA available to regulate its mRNA targets. The expression of *fliA*, which is regulated by FlhDC, a CsrA target that was shown to be stabilized by the regulatory protein CsrA, is downregulated under our conditions. Therefore, for all of the CsrA targets tested, the relative expression is similar to what is described in literature for a *csrB* overexpression strain.

Subsequently, it was hypothesized that the differences in biofilm formation might be explained by the conditions under which the biofilms were grown. Indeed, for most of the biofilm experiments done by Jackson and coworkers (2002), the strains were grown in CFA at 37°C, while we tested the biofilm forming capacity of the strains in 1/20 TSB at 25°C. To validate this hypothesis, the biofilm forming capacity of *E. coli* cells overexpressing known CsrA targets in 1/20 TSB at 25°C and in CFA at 37°C was evaluated. Because the qRT-PCR results shown in **Figure 3.10** indicate that *ycdT* and *ydeH* expression is highly upregulated when the complete *csrB* sequence is expressed, studying the effect of overexpressing these genes on biofilm development was given priority. The results are shown in **Figure 3.11**.

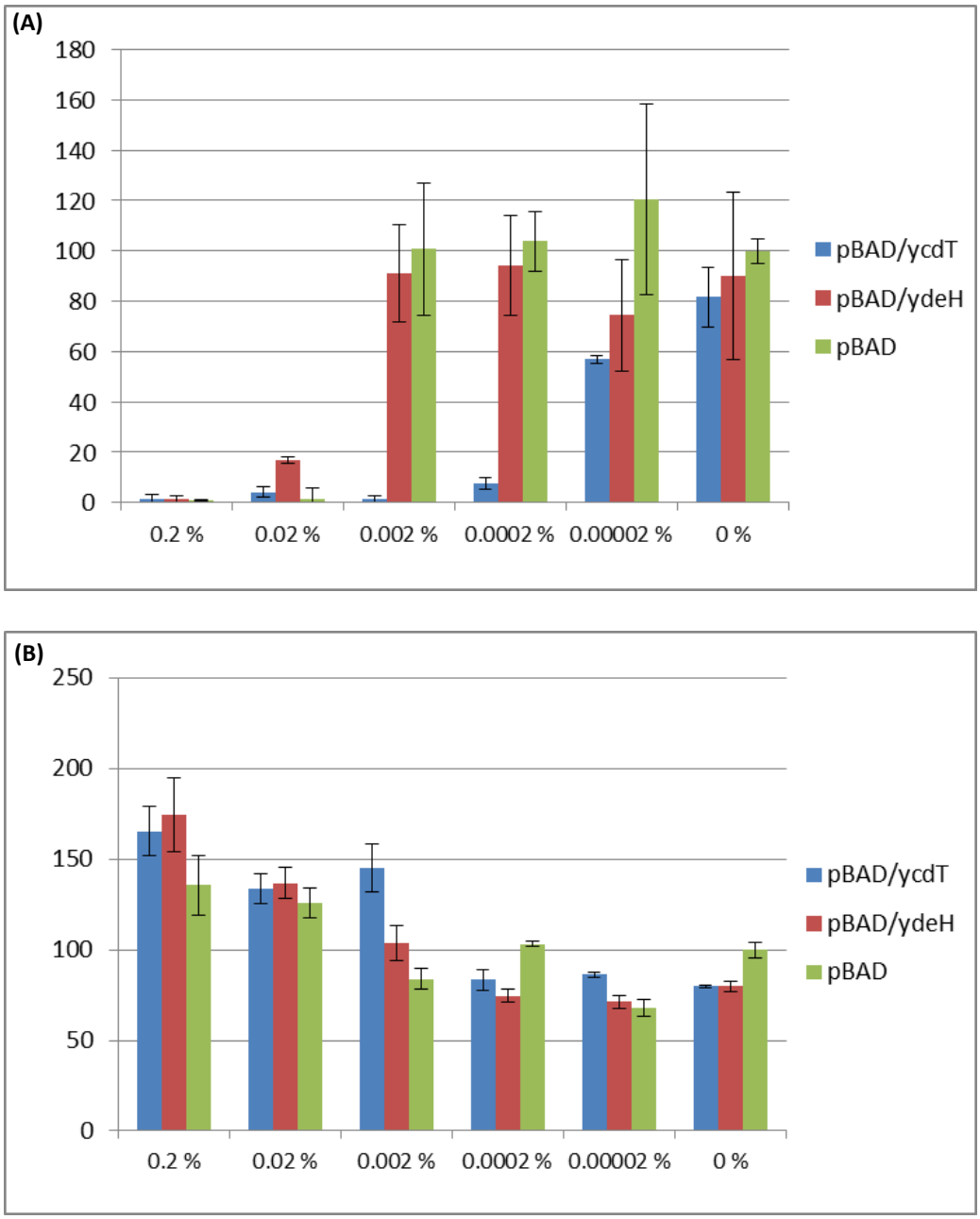


Figure 3.11 Biofilms formed by *E. coli* DH5α strains expressing *ycdT* or *ydeH* from an arabinose inducible promoter, induced with different concentrations of arabinose (A) when grown in 1/20 TSB at 25°C and **(B)** when grown in CFA at 37°C. Biofilm formation was measured after 48 hours of growth. Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid grown in the absence of arabinose is set at 100 %. The results are representative for three biological repeats, in which every strain was tested in three technical repeats.

In **Figure 3.11 (A)**, the results are shown for strains grown in 1/20 TSB at 25°C. For strains grown in this medium, there is a concentration-dependent negative effect of *ycdT* expression on biofilm formation. Remarkably, arabinose also affects the biofilm forming capacity of the bacteria when grown in this medium, as can be observed from the data of the strain with an empty pBAD vector. However, inducing the expression of *ycdT* with 0.002 % arabinose reduces the biofilm forming capacity of *E. coli*, while this concentration of arabinose did not have an effect on biofilm formation of the control strain. Overexpressing *ydeH* has no effect on the biofilm forming ability of cells grown in 1/20 TSB at 25 °C when compared to strains with an empty pBAD vector. The reduced amount of biofilm that was detected in strains overexpressing *ycdT* when grown in 1/20 TSB at 25 °C, could not be observed when the cells were grown in CFA at 37 °C, shown in **Figure 3.11 (B)**. The biofilm results suggest that under these conditions, there are in general no large differences in the biofilm forming capacity of strains overexpressing *ycdT*, *ydeH* or strains with an empty vector. Therefore, the condition-dependent effect of overexpressing *ycdT*, one of the targets of CsrA, on biofilm development might be one of the reasons why a *csrB* overexpression strain is reduced in biofilm forming capacity in 1/20 TSB, whereas it was reported otherwise in literature (Jackson *et al.*, 2002).

3.4. Discussion

CsrA has been shown to post-transcriptionally regulate the expression of a large number of genes, some of which are known to be involved in biofilm development (Jackson *et al.*, 2002; Pannuri *et al.*, 2012; Wang *et al.*, 2005). The activity of this protein is regulated by different sRNAs. One of these CsrA regulating sRNAs is CsrB, which was shown to be involved in the regulation of biofilm formation as well (Jackson *et al.*, 2002). In this chapter, sequences were designed to interfere with CsrA or CsrB function, as it was speculated that these sequences could disturb the process of biofilm development.

To interfere with CsrA or CsrB function, different strategies were tested. Firstly, the complete reverse complementary *csrB* sequence and short sequences that are the reverse complement of different regions within CsrB resembling the CsrA binding site, were expressed. Base pairing between CsrB and these molecules was predicted to prevent the regulatory action of CsrB. The presence of a specific band indicated that the sequence was expressed under biofilm conditions. However, multiple bands

are visible on the blot. This is probably due to some non-specific binding of the relatively small probe, as these bands were also detected in the RNA sample of cells expressing the random sequence. A blast search revealed that the probe might bind to other transcripts with similar binding energies: in the 3' region of the zink-dependent transcriptional repressor *zur* mRNA (516 bp), in the coding region of the CPZ-55 prophage *yffM* mRNA (246 bp) and in the coding region of the 4-aminobutyrate aminotransferase *gabT* mRNA (1281 bp). Probably other (processed) transcripts are detected as well, as there is a band, indicative for a transcript of less than 100 bp, which could not be explained based on the results of the blast search. As the density of the non-specific bands in the random sample is lower, this suggests that either less RNA was loaded or expression of the sequence has an effect on the expression of these non-specific targets, which is unlikely.

Based on literature data, which described that an *E. coli ΔcsrB* mutant was impaired in biofilm development (Jackson *et al.*, 2002), we expected that expressing these antisense sequences would reduce the biofilm forming capacity of the strain. However, none of the expressed antisense sequences reduced the amount of biofilm formed on the pegs of the Calgary Biofilm Device when the cells were grown in 1/20 TSB at 25°C. It is unclear why even the antisense sequence comprising the reverse complement of the complete *csrB* sequence did not affect biofilm development, as this approach has been used before to deplete sRNAs (Kint *et al.*, 2010). Possibly the extensive secondary structure of CsrB and the antisense sequence hampers their interaction. Therefore, it might be interesting to include a Hfq binding site in the antisense sequence, as for some natural sRNA-mRNA interactions, Hfq has been shown to induce a conformational change, enabling the sRNA and the mRNA to interact (Henderson *et al.*, 2013; Soper *et al.*, 2011). Additionally, Hfq can stabilize sRNAs, increasing the concentration of this sequence in the cell (Valentin-Hansen *et al.*, 2004). An Hfq binding site might consequently increase the efficiency of the interaction between CsrB and the antisense CsrB sequence. However, sRNAs that are extensively complementary to their target, like *cis*-encoded sRNAs, have been shown to function in an Hfq-independent way (Brantl, 2007). As CsrB and the *csrB* antisense sequence are also highly complementary, including an Hfq binding site might only have a small effect, all the more because CsrB action is Hfq-independent (Jørgensen *et al.*, 2013). Alternatively, it is possible that a reduced CsrB activity does not affect biofilm formation under the conditions tested or that other sRNAs with the same function take over the role of CsrB in biofilm regulation.

Secondly, the complete *csrB* sequence and short sequences that resemble the regions within CsrB that mimic a CsrA binding site and are responsible for interaction with CsrA, were expressed. The overexpression of the complete sense *csrB* sequence reduces biofilm formation on the pegs of the

Calgary Biofilm Device and affects growth in 1/20 TSB at 25°C. Additionally, expressing the sense stem loop stem sequence, of which the sequence is based on the regions of CsrB interacting with CsrA, mimicking the CsrA binding sequence, was also shown to affect biofilm formation but did not affect growth. In **Chapter 4**, the mode of action of this interesting short sequence will be studied in more detail.

Additionally, mirror sequences were designed and tested for their effect on biofilm formation. The inclusion of a mirror sequence in a study was done before (Tchurikov *et al.*, 2000). Based on the work of Tchurikov and coworkers, no effect on biofilm development was expected. However, the amount of biofilm formed in the presence of the mirror stem loop stem sequence is significantly lower compared to strains with an empty plasmid and also the growth of the strain is affected when this sequence is expressed. The mirror stem loop stem sequence will be characterized and its mode of action will be analyzed in **Chapter 6**. We do not expect this sequence to affect regulators in the CsrA-based regulatory network.

The observation that the overexpression of *csrB* reduces biofilm formation was in contrast with previously published results that showed that overexpressing *csrB* induced biofilm formation (Jackson *et al.*, 2002). We hypothesize that this is caused by a condition-dependent effect of some genes within the CsrA-based regulatory network. We were able to show that the upregulation of a specific CsrA target, YcdT, which is a transmembrane protein that possess diguanylate cyclase activity, affects biofilm formation differently depending on the condition (medium/temperature) in which the bacteria were grown. When grown in 1/20 TSB at 25 °C, representative for conditions outside the host like on industrial surfaces, YcdT has a negative effect on biofilm development, while in CFA at 37 °C, representative for conditions inside the host, no effect on this phenotype could be observed. Earlier phenotypic characterizations indeed revealed that biofilm formation was not affected by YcdT when grown in LB or CFA at 26 °C (Wang *et al.*, 2005). Conversely, the protein was shown to be involved in the regulation of motility, as overexpression of *ycdT* leads to reduced swimming behavior. Mutations in its GGDEF domain suggest that this is a c-di-GMP-dependent effect (Jonas *et al.*, 2008). When grown in LB at 37°C, c-di-GMP levels are slightly upregulated in a *ΔcsrA* mutant. This might be different when cells are grown in poor medium.

Because YcdT was described not to have an effect on biofilm formation when grown in LB or CFA at 26°C (Wang *et al.*, 2005) and we do observe an effect when cells are grown in 1/20 TSB at 25°C, this suggests that it is the growth medium that makes the difference. It has been shown before that when grown in different media, there is differential gene expression, including for numerous genes

encoding products associated with the cell surface or the cell membrane, which are important for adherence and biofilm formation (Roos *et al.*, 2005). Therefore, the biofilm forming capacity of a strain in one medium has been described to provide no clue about its performance in another medium (Hancock *et al.*, 2011).

Although the effect of YcdT is probably medium-dependent, other genes in the CsrB-CsrA network might have a condition-dependent effect as well. This condition-dependency might not only relate to the growth medium, but also to the temperature. Biofilm formation and adherence are not accomplished by the same mechanism when biofilms are grown under different temperatures, just like when grown in different media. For instance, at 37°C, the matrix mainly consists of PGA and sometimes also curli and cellulose. Attachment is mediated by type I fimbriae and Ag43. At ambient temperature (below 30°C) it are curli, cellulose and also colonic acid and flagella that dominate matrix composition and also mediate surface attachment (Mika & Hengge, 2014). This different matrix composition when grown under different temperatures can be attributed to the induction of RpoS transcription at low temperatures, leading to increased levels of CsgB, CsgA and CsgD (White-Ziegler *et al.*, 2008). Additionally, under low temperatures, Crl recruits σ^S bound polymerase to the promoter of *csgBA* (Bougdour *et al.*, 2004). Because of the condition-dependent differences in biofilm development, it is important that the activity of biofilm inhibitors are tested under different conditions.

Remarkably, our experiments also showed that arabinose affects biofilm development when cells are grown in 1/20 TSB at 25°C. Although this effect has not been described in detail in literature, high levels of sugars can increase the osmolarity of the medium, which is inhibitory to biofilm formation (Prigent-Combaret *et al.*, 2001). Additionally, sugars in general have been shown to activate biofilm growth at low concentrations and inhibit it at high concentrations (Sutrina *et al.*, 2015). Acidification due to fermentation of the sugar was shown to attribute to this inhibitory effect.

CHAPTER 4

THE SENSE SEQUENCE

Analysis of a sequence that mimics the CsrA binding sequence

In this chapter, focus is on the biofilm reducing sense stem loop stem sequence, identified in **Chapter 3**. An analysis of the nucleotide requirements and a gene expression study in the presence of the sense stem loop stem confirm that this sequence sequesters CsrA, as it was designed to do. Additionally, the effect of expressing the sense stem loop stem sequence on biofilm formation was evaluated in an *E. coli* TG1 strain and in another species, *Salmonella enterica* serovar Typhimurium. In *E. coli* TG1, expressing the sense stem loop stem sequence negatively affects biofilm development as well. In *S.* Typhimurium, however, expressing this sequence has no effect on the capacity to form biofilms.

4.1. Introduction

In the previous chapter, two interesting short sequences that reduce biofilm formation when expressed from a plasmid in *E. coli* DH5α, were identified. Here, we will focus on one of these sequences, the sense stem loop stem sequence.

By mimicking the regions within CsrB responsible for interaction with CsrA, the sense stem loop stem sequence is thought to sequester CsrA, reducing its activity. Consequently, CsrA cannot regulate the expression of its mRNA targets, thereby disturbing the processes that are controlled by the protein, such as biofilm formation. Here, we will test whether the negative effect of the short sense stem loop stem sequence on biofilm formation is indeed caused by disturbing the CsrA-based regulatory network.

Additionally, the effect of expressing the stem loop stem sequence will be evaluated in *E. coli* TG1 and in *Salmonella* Typhimurium. In *S.* Typhimurium, both CsrA and CsrB show strong sequence homology to their counterparts in *E. coli* (Altier *et al.*, 2000a; Lapouge *et al.*, 2008). However, the target genes that are regulated vary between *E. coli* and *S.* Typhimurium. While CsrA is a global regulator in *E. coli*, the protein seems to be involved in the regulation of more specific processes in *S.* Typhimurium, such as the regulation of invasion (Altier *et al.*, 2000b; Lawhon *et al.*, 2003; Martínez *et al.*, 2011). Nonetheless, the Csr-system was shown to be involved in the regulation of biofilm formation in *S.* Typhimurium as well (Teplitski *et al.*, 2006).

4.2. Materials and methods

4.2.1 Bacterial strains and growth conditions

Bacteria were grown as described in **section 3.2.1**. The bacterial strains and plasmids used in this chapter are listed in **Table 4.1** and **4.2**, respectively.

Table 4.1 Bacterial strains

Strain name	Description	Reference
***E. coli* DH5α**	F⁻ϕ80Δl*acZM15*Δ(l*acZYAargF*)U169 *deoP recA1endA1 hsdR17* (rk⁻ mk⁻)	Gibco BRL
***E. coli* TG1**	F' *Δlac pro supE thi hsdD5 traD36 proAB+, lacIq lacZΔM15*	Amersham Pharmacia Biotech
***E. coli* TOP10**	F´ {*lacIq* Tn10(TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80*lacZΔM15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (*StrR*) *endA1 nupG*	Invitrogen
***S.* Typhimurium SL1344**	*xyl, hisG, rpsL*; virulent; Sm^R	Hoiseth & Stocker, 1981

Table 4.2 Plasmids

Plasmid name	Description	Reference
pJV853-1	Anti-*micA* expression construct based on pZE12-luc. P_{LlacO} promoter, Ap^R	Gift from J. Vogel, published in (Kint *et al.*, 2010)
pCMPG10905	pJV853-1 based plasmid, expressing sense stem loop stem, constructed with PRO-8349 and S&P-00165, *EcoRI* restriction site	Chapter 3
pCMPG10907	pJV853-1 based plasmid, expressing random sequence, constructed with PRO-7172 and PRO-7573, *PstI* restriction site	Chapter 3
pCMPG10908	pJV853-1 based control plasmid, no sequence incorporated, constructed with PRO-7171 and PRO-7172, *PstI* restriction site	Chapter 3
pCMPG10909	pJV853-1 based plasmid, expressing complete CsrB sequence (amplified with S&P-00410 and S&P-00411), constructed with PRO-7171 and PRO-8349, *EcoRI* restriction site	Chapter 3
pCMPG10913	pJV853-1 based plasmid, expressing sense stem loop stem, constructed with PRO-7172 and S&P-00412, *PstI* restriction site	This work
pCMPG10932	pJV853-1 based plasmid, expressing mutated sense stem loop stem, constructed with S&P-00907 and PRO-7172 (mutated AGGA -> ATTA), *PstI* restriction site	This work
pCMPG10933	pJV853-1 based plasmid, expressing mutated sense stem loop stem, constructed with S&P-00909 and PRO-8349 (mutated AGGA -> ATTA), *EcoRI* restriction site	This work

4.2.2 Construction of plasmids

Plasmids expressing the short sequences were constructed as described in **section 3.2.2**. The primers that were specifically used in this chapter are listed in **Table 4.3**. Plasmids were transferred form *E. coli* DH5α to *E. coli* TG1 by transformation and to *S.* Typhimurium by electroporation.

Table 4.3 Primers

Primer	Sequence	Purpose	
PRO-7172	ATCTGCAGGGCTCAGTCGAAAGACTG	Rv	Every pJV853-1 based plasmid with *PstI* restriction site
PRO-8349	ATGAATTCGGCTCAGTCGAAAGACTG	Rv	Every pJV853-1 based plasmid with *EcoRI* restriction site
S&P-00412	ATCTGCAGACATCCTTGTAC GTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10913
S&P-00907	ATCTGCAGACATAATTGTACGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10932
S&P-00909	ATGAATTCACATAATTGTAC GTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10933

4.2.3 RNA isolation, cDNA synthesis and qRT-PCR analysis

RNA samples were taken as described in **section 3.2.3**. cDNA was made and qRT-PCR analyses were done according to the method described in **section 3.2.5**. The primers used for qRT-PCR analysis are listed in **Table 3.5**.

4.2.4 Biofilm and growth assay

Biofilms were grown and growth assays were done as described in **section 3.2.6**. and **3.2.7**, respectively.

4.2.5 DNA and protein alignment

DNA and protein sequences were retrieved from NCBI. Sequences were aligned using the Clustal omega analysis tool (Goujon *et al.*, 2010), available at http://www.ebi.ac.uk/Tools/msa/clustalo/.

4.3. Results

4.3.1 Characterization of the sense stem loop stem sequence

A first step in the validation of the mode of action of the sense stem loop stem sequence was to get more insight into the nucleotides in the sequence that are important for the negative effect on biofilm development. Firstly, the effect of the restriction site in the plasmid that encodes the sense stem loop stem was studied. Therefore, an additional sense stem loop stem construct was made, containing a *PstI* instead of an *EcoRI* restriction site. The latter restriction site is present in the plasmids that were tested in **Chapter 3**. Subsequently, the biofilm forming capacity of strains expressing the sense stem loop sequence from a construct with a *PstI* site was tested. The result is shown in **Figure 4.1**.

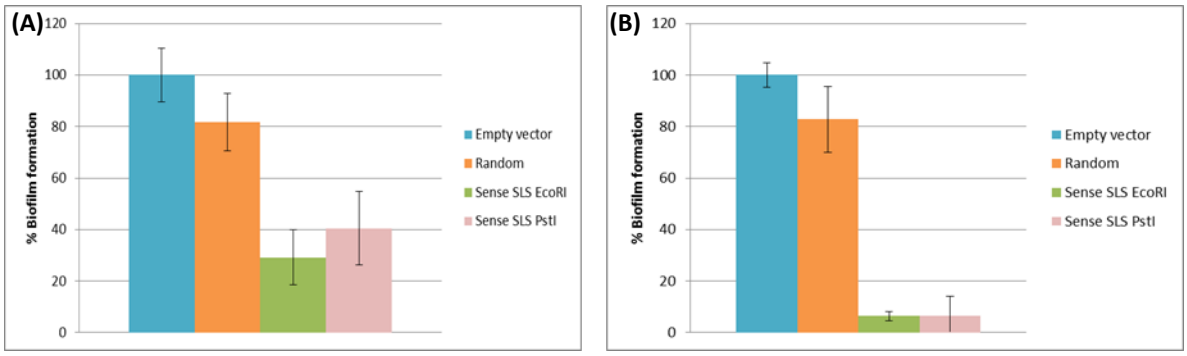


Figure 4.1 Biofilms formed by *E. coli* DH5α strains with plasmids expressing the sense stem loop stem sequence from a plasmid constructed with different restriction sites (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), short sense stem loop stem with an *EcoRI* restriction site (sense SLS *EcoRI* - pCMPG10905), short sense stem loop stem with a *PstI* restriction site (Sense SLS *PstI* - pCMPG10913). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

The results in **Figure 4.1** show that expressing the sense stem loop stem sequence from both constructs (pCMPG10905 and pCMPG10913) significantly reduces the biofilm forming ability of *E. coli* DH5α. Consequently, it can be concluded that the nucleotides of the restriction site are not important for the effect on biofilm formation. As nucleotides of the restriction site do not overlap

with the consensus CsrA binding site, this is in agreement with the hypothesis that the sequence sequesters CsrA.

Secondly, the GGA nucleotides in the sense stem loop stem sequence were mutated. It has been described in literature that these nucleotides, typically present in the mRNA targets of CsrA and in the loops of CsrB, are essential for binding to CsrA (Dubey *et al.*, 2005). Therefore, the GGA in the short sense stem loop stem sequence was mutated to TTA, both in the construct with an *EcoRI* restriction site and in the construct with a *PstI* restriction site. The results are shown in **Figure 4.2**.

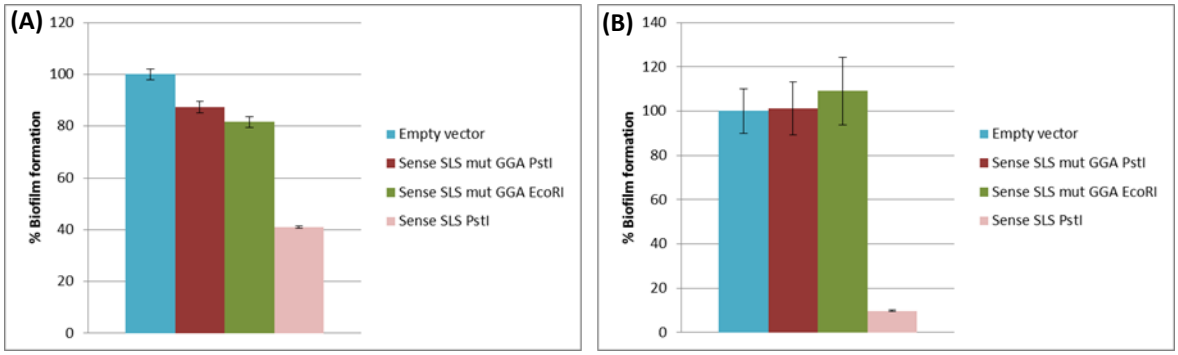


Figure 4.2 Biofilms formed by *E. coli* DH5α strains with plasmids expressing the sense stem loop stem sequence and a mutated form of this sequence (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing the mutated (Sense SLS mut GGA PstI - pCMPG10932 and Sense SLS mut GGA EcoRI - pCMPG10933) and original short sense stem loop stem sequence (Sense SLS PstI - pCMPG10913). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

Figure 4.2 shows that mutating the GGA to TTA in the sense stem loop stem sequence (pCMPG10932 and pCMPG10933) results in a restoration of biofilm development, comparable to the amount of biofilm formed by strains with the empty plasmid (pCMPG10908). These results demonstrate that the GGA nucleotides are essential for the action of the short sense stem loop stem sequence. As these GGA nucleotides are also very important in a CsrA binding sequence, this supports the hypothesis that the short sense stem loop stem sequence is acting through sequestering CsrA.

4.3.2 CsrA target gene expression analysis

To further confirm the hypothesis that the stem loop stem sequence affects the CsrA-based regulatory network by sequestering CsrA, an expression analysis was performed. Because the short sense stem loop stem sequence is thought to affect CsrA activity, the expression levels of some known CsrA targets, the same genes that were tested in **section 3.3.4**, were measured in the presence of the short sense sequence (pCMPG10905). Hereto, RNA samples were taken from cells grown in 1/20 TSB at 25°C under shaking conditions. The samples were taken in exponential phase of planktonic growth (OD 0.1) and not in biofilm phase, because the sequence affects biofilm formation and therefore it is difficult to find the time point at which the strain with an empty vector and the strain expressing the sense stem loop stem sequence have the same growth phase-dependent background gene expression. Moreover, if the CsrA activity is reduced, this should also be observed under planktonic growth. Additionally, the results in **Figure 3.10** indicate that in early exponential phase in 1/20 TSB (OD 0.1) *ycdT*, *ydeH*, *glgC* and *pgaA* expression is upregulated when *csrB*, which functions as a positive control and is known to downregulate CsrA activity, is expressed (see **Figure 4.3 (A)**). The qRT-PCR results for cells grown under these conditions in the presence of the sense stem loop stem sequence are shown in **Figure 4.3 (B)**.

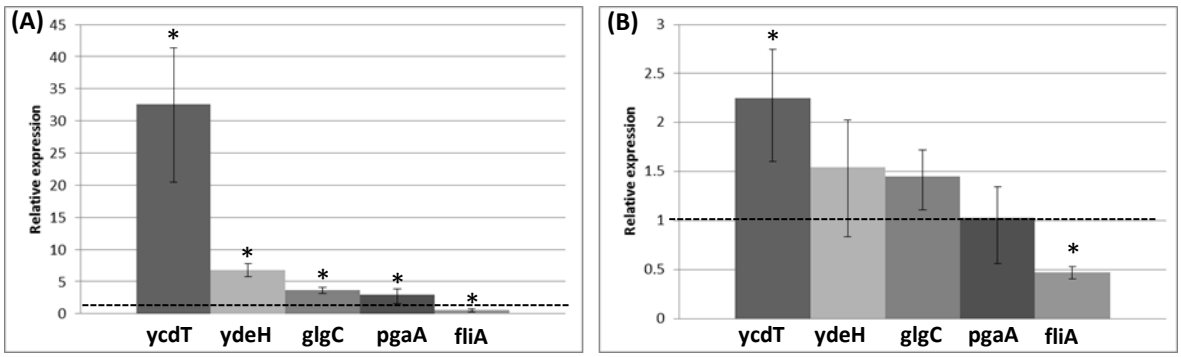


Figure 4.3 Relative expression levels of a selected number of CsrA targets in cells (A) with the *csrB* overexpression plasmid (pCMPG10909) and (B) with the short sense stem loop stem plasmid (pCMPG10905). The expression levels are relative to the expression levels in cells with a control plasmid (pCMPG10908). Cells were grown shaking in 1/20 TSB at 25°C. Error bars represent 95% confidence intervals. A star indicates that the C_T values are significantly different for both strains ($p < 0.05$). The dotted line indicates a relative expression level of 1. Relative expression levels > 1 indicate increased expression, relative expression levels <1 indicate reduced expression compared to the control strain.

If CsrA activity is reduced, an upregulation in the expression of the CsrA targets that are negatively regulated by CsrA and a downregulation in the expression of the CsrA targets that are positively regulated is expected, similar to what was observed for the strain that overexpresses *csrB* (see **Figure 4.3 (A)**). The results in **Figure 4.3 (B)** show that the expression of *ycdT* is indeed upregulated in the presence of the sense stem loop stem sequence, while the expression of *fliA* is downregulated compared to the expression levels in the strain with the empty plasmid. The expression levels of *ydeH* and *glgC,* although not significantly different from the expression levels in strains with the empty plasmid, also show the same trend as the strains that overexpresses *csrB* (see **Figure 4.3 (A)**) .

Additionally, the expression levels of the CsrA targets in cells grown in LB were measured. If the short sense stem loop stem is reducing CsrA activity, an effect on the CsrA target expression should be observed in this medium as well. RNA samples were taken from exponentially growing cells (OD 1). The results are shown in **Figure 4.4**.

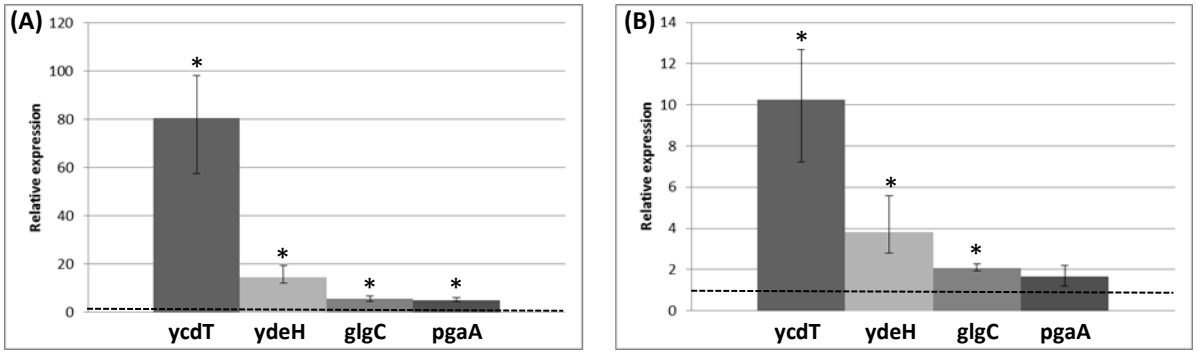


Figure 4.4 Relative expression levels of a selected number of CsrA targets in cells (A) with the CsrB overexpression plasmid (pCMPG10909) and (B) with the short sense stem loop stem plasmid (pCMPG10905). The expression levels are relative to the expression levels in cells with a control plasmid (pCMPG10908). Cells were grown in LB at 25°C. Error bars represent 95% confidence intervals. A star indicates that the C_T values are significantly different for both strains ($p < 0.05$). The dotted line indicates a relative expression level of 1. Relative expression levels > 1 indicate increased expression, relative expression levels <1 indicate reduced expression compared to the control strain.

In **Figure 4.4 (A)** the qRT-PCR results are shown for cells that express the complete *csrB* sequence (pCMPG10909) when grown in LB. Also under these conditions *csrB* overexpression resulted in the expected upregulation of *ycdT*, *ydeH*, *glgC* and *pgaA* expression when compared to the expression level in cells with an empty plasmid. The results in **Figure 4.4 (B)** show that *ycdT*, *ydeH*, *glgC* and

pgaA expression is upregulated as well when the short sense stem loop stem is expressed in cells grown in LB (pCMPG10905). The relative differences are higher than what was observed when the same strains were grown in 1/20 TSB and strengthen the hypothesis that the short sense stem loop stem sequence is sequestering CsrA (see **Figure 4.3**).

The relative differences in gene expression observed for strains expressing the sense stem loop stem sequence (pCMPG10905) are smaller than for strains overexpressing the complete *csrB* sequence (pCMPG10909), when grown either in 1/20 TSB (see **Figure 4.3**) or in LB (see **Figure 4.4**). This suggests that the short sequence is less efficient than the complete CsrB sequence and might be improved by enlarging the sequence with an additional CsrA binding site or a stabilizing element. However, because our goal is to find short sequences that can be applied as a biofilm inhibitor, this was not studied further.

4.3.3 Expanding the analysis to *E. coli* TG1 and *S.* Typhimurium

All previous experiments were done with *E. coli* DH5α, because most information about CsrA, CsrB and their role in the regulation of biofilm development is available for *E. coli*. Additionally, DH5α is an *E. coli* strain that forms a significant amount of biofilm in 1/20 TSB in the Calgary Biofilm Device. Here, the effect of the sense stem loop stem sequence (pCMPG10905 and pCMPG10913) on biofilm development in another *E. coli* strain, TG1, was examined. The results of the biofilm test are shown in **Figure 4.5**.

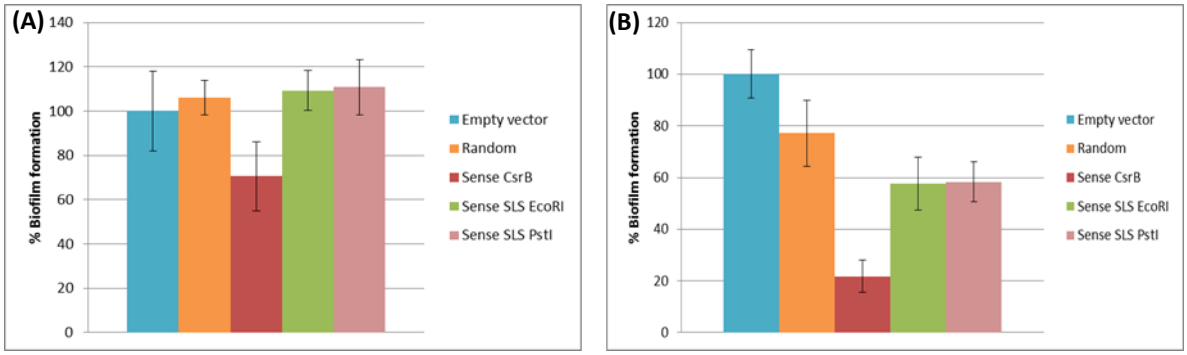


Figure 4.5 Biofilms formed by *E. coli* TG1 strains with plasmids expressing the sense stem loop stem sequence from a plasmid constructed with different restriction sites (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), sense CsrB (pCMPG10909), short sense stem loop stem with an *EcoRI* restriction site (pCMPG10905), short sense stem loop stem with a *PstI* restriction site (pCMPG10913). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

The results in **Figure 4.5** show that for *E. coli* TG1, there is a negative effect on the biofilm forming capacity when bacteria express the complete *csrB* sequence (pCMPG10909). Also expressing the sense stem loop stem sequence (pCMPG10905 and pCMPG10913) reduces the amount of biofilm that is formed after 48 hours of incubation. However, the effect is smaller than observed in DH5α, both for strains expressing the complete *csrB* sequence and the short sense stem loop stem sequence.

The Csr-system is widely distributed among eubacterial cells and the sequences of *csrA* and *csrB* of *E. coli* and *S.* Typhimurium show strong homology as illustrated by the DNA and protein alignments shown in **Figure 4.6** and **4.7**. The DNA sequence of CsrA shows eight different nucleotides on DNA level, but because these are synonymous changes, the sequence is identical on protein level, suggesting identical RNA binding characteristics. For CsrB, the RNA sequence shows a larger difference than for CsrA, but the GGA, typical for CsrA binding sites is conserved between the sequence in *E. coli* and *S.* Typhimurium.

(A) CLUSTAL O(1.2.1) multiple sequence alignment

```
E_coli_MG1655          ATGCTGATTCTGACTCGTCGAGTTGGTGAGACCCTCATGATTGGGGATGAGGTCACCGTG     60
S_Typhimurium_LT2      ATGCTGATTCTGACTCGTCGAGTTGGTGAGACCCTCATGATTGGCGATGAGGTCACCGTG     60
                       ******************************************** ***************

E_coli_MG1655          ACAGTTTTAGGGGTAAAGGGCAACCAGGTACGTATTGGCGTAAATGCCCCGAAGGAAGTT     120
S_Typhimurium_LT2      ACAGTTTTAGGGGTGAAGGGCAACCAGGTGCGTATTGGCGTGAACGCCCCGAAAGAAGTT     120
                       ************** ************** *********** ** ******** ******

E_coli_MG1655          TCTGTTCACCGTGAAGAGATCTACCAGCGTATCCAGGCTGAAAAATCCCAGCAGTCCAGT     180
S_Typhimurium_LT2      TCTGTCCATCGTGAAGAGATCTACCAGCGTATCCAGGCTGAAAAATCCCAGCAGTCCAGT     180
                       ***** ** ************************************************

E_coli_MG1655          TACTAA   186
S_Typhimurium_LT2      TACTAA   186
                       ******
```

(B) CLUSTAL O(1.2.1) multiple sequence alignment

```
E_coli_MG1655          mliltrrvgetlmigdevtvtvlgvkgnqvrigvnapkevsvhreeiyqriqaeksqqss     60
S_Typhimurium_LT2      mliltrrvgetlmigdevtvtvlgvkgnqvrigvnapkevsvhreeiyqriqaeksqqss     60
                       ************************************************************

E_coli_MG1655          y        61
S_Typhimurium_LT2      y        61
                       *
```

Figure 4.6 Alignment of (A) the DNA sequence and (B) the protein sequence of *csrA* in *E. coli* MG1655 and *S.* Typhimurium LT2 using Clustal omega.

```
CLUSTAL O(1.2.1) multiple sequence alignment

E_coli_MG1655          GTCGACAGGGAGTCAGACAACGAAGTGAACATCAGGATGATGACACTTCTGCAGGACACA     60
S_Typhimurium_LT2      --------GAGTCGTACAACGAAGCGAACGTCAGGATGATGACGCTTCAGCAGGACACG     51
                               *****  ********* **** ************* **** *********

E_coli_MG1655          CCAGGATGGTGTTTCAGGGAAAGGCTTCTGGATGAAGCGAAGAGGATGACGCAGGACGCG     120
S_Typhimurium_LT2      CCAGGATGGTGTTACAAGGAAAGGCTTCAGGATGAAGCAAAGTGGAAAGCGCAGGATGCG     111
                       ************* ** *********** ********* *** ***   ******* ***

E_coli_MG1655          TTAAAGGACACCTCCAGGATGGAGAATGAGAACCGGTCAGGATGATTCGGTGGGTCAGGA     180
S_Typhimurium_LT2      TTAAAGGACACCTCCAGGACGGAGAACGAGAGCCGATCAGGATGTTCGG-CGGGTCTGGA     170
                       ****************** ****** **** *** ******** *  *  ***** ***

E_coli_MG1655          AGGCCAGGGACACTTCAGGATGAAGTATCACATCGGGGTGGTGTGAGCAGGAAGCAATAG     240
S_Typhimurium_LT2      TGACCAGGGACGCTTCAGGAAGAAGCTATCACATCGGGCGATGTGCGCAGGATGCAAACG     230
                        * ******** ******** ****          *** * **** ****** ****  *

E_coli_MG1655          TTCAGGATGAACGATTGGCCGCAAGGCCAGAGGAAAAGTTGTCAAGGATGAGCAGGGAGC     300
S_Typhimurium_LT2      TTCAGGATGA---ACAGGCCGTAAGGTCACAGGAAAAGTTGTCACGGATGAGCAGGGAGC     287
                       **********   *  ***** **** ** ************** ***************

E_coli_MG1655          AACAAAAGTAGCTGGAATGCTGCGAAACGAACCGGGAGCGCTGTGAATACAGTGCTCCCT     360
S_Typhimurium_LT2      ACGAAAAGTAGCTGGAATGCTGCGAAACGAACCGGGAGCACTGTTTATACAGTGCTCCCT     347
                       *  ************************************ ****  ***************

E_coli_MG1655          TTTTTTATT        369
S_Typhimurium_LT2      TTTTTTTG-        355
                       ******
```

Figure 4.7 Alignment of the DNA sequence of *csrB* in *E. coli* MG1655 and *S.* Typhimurium LT2 using Clustal omega. All the RGGA motifs present in the sequence, characteristic for a CsrA binding site, are framed.

Because of this high conservation, the effect of expressing the short sense stem loop stem sequence (pCMPG10905 and pCMPG10913) and the complete CsrB sequence (pCMPG10909) on biofilm development was also tested in *S.* Typhimurium (see **Figure 4.8**). In *S.* Typhimurium, expressing *csrB* does not affect the biofilm capacity of the strain, nor is the short sense stem loop stem sequence.

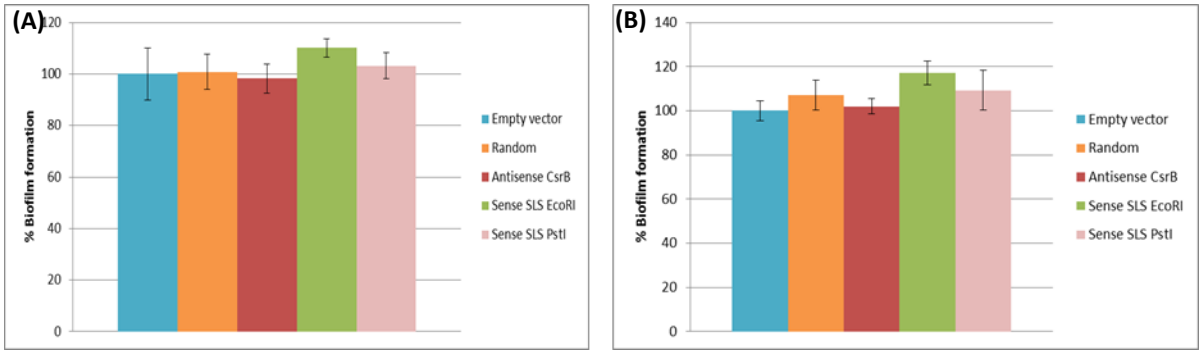


Figure 4.8 Biofilms formed by *Salmonella* Typhimurium SL1344 strains with plasmids expressing the sense stem loop stem sequence from a plasmid constructed with different restriction sites (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), sense CsrB (pCMPG10909), short sense stem loop stem with an *EcoRI* restriction site (pCMPG10905), short sense stem loop stem with a *PstI* restriction site (pCMPG10913). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

4.4. Discussion

The biofilm reducing sense stem loop stem sequence was designed in **Chapter 3** to interfere with CsrA activity. The sequence is based on the regions within CsrB that interact with CsrA, which resemble the consensus CsrA binding site, and is therefore thought to act as an RNA decoy, sequestering CsrA, similar to the action of CsrB. RNA decoys were already tested in eukaryotes, concluding that rational design and testing can result in decoy development with physiological consequences (Makeyev *et al.*, 2002).

To validate its mode of action, the sense stem loop stem sequence was characterized, showing that the restriction site, which does not overlap the consensus binding site, is not important for the effect

of the sequence on biofilm development. Additionally, the GGA in the sequence is shown to be essential for its biofilm inhibitory effect, which fits with the requirements for binding CsrA. These observations support the hypothesis that the sense stem loop stem sequence affects biofilm development through sequestering CsrA. Moreover, a gene expression analysis showed that CsrA targets, known to be downregulated by CsrA, are upregulated in the presence of the sense stem loop stem sequence and vice versa, similar to what was observed for cells overexpressing *csrB*, which is a positive control as it is known to reduce CsrA activity. This suggests that CsrA activity is also reduced in the presence of the sense stem loop stem sequence and confirms the hypothesis that the sense stem loop stem sequence sequesters CsrA. The differences in gene expression when overexpressing the complete *csrB* sequence or the short sense stem loop stem sequence are larger when the cells were grown in LB as compared to cells grown in 1/20 TSB. It might be that expression of the short sequences is more stable in rich medium. Another possibility is that in 1/20 TSB, the mRNA targets of CsrA are present at a different abundance, which might cause less CsrA to be available to regulate the targets that were tested here, whereby these show smaller expression differences.

The short sense stem loop stem sequence has similar molecular and phenotypic effects compared to the sRNA CsrB. This suggests that expressing a sequence that resembles the target interaction region of a sRNA, in this case a CsrA binding sequence, is sufficient for the regulatory effect of the sRNA. Moreover, as the sense stem loop stem sequence is small, it is more interesting as the sequence needs to be taken up by the cells when it is used as a biofilm inhibitor. However, both the effect on biofilm development and the effect on gene expression is smaller for the short sequence than for the complete CsrB sequence, suggesting that the short sense stem loop stem sequence is less efficient than CsrB. This is probably because CsrB contains multiple sites resembling the CsrA binding sequence. Increasing the plasmid-based expression of the short sequence might increase the effect on gene expression and on biofilm development. However, the promoter on the plasmid is maximally induced with 1 mM IPTG. Furthermore, the efficiency of the sequence, with respect to the downregulation of CsrA activity and the reduction of biofilm development, might be increased by the extension of the sequence with an additional CsrA binding site, because CsrA is homodimer (Dubey *et al.*, 2003), capable of binding two neighboring CsrA binding sites in a single target (Mercante *et al.*, 2010). Additionally, increasing the stability of the short sequence might increase its efficiency. This can be done by the incorporation of flanking sequences of inverted repeats that form paired termini (Nakashima *et al.*, 2006). Additionally, in synthetic sequences, an Hfq binding site is often included, as natural sRNAs often depend on Hfq for their stability (Sauer, 2013b) and an Hfq binding site can additionally promote the interaction with the targets of the sRNA and promote the degradation of

the mRNA target (Na *et al.*, 2013; Sharma *et al.*, 2012). However, the inclusion of an Hfq binding site was done for sequences that base pair with their target, while CsrB interacts with proteins and is not dependent on Hfq for its stability or its interaction with CsrA. Therefore, it is unclear if this inclusion would indeed increase the efficiency of the sequence. Although these proposed strategies might increase the stability of the plasmid expressed sequences, because of size constraints, these elongated sequences (additional CsrA binding sites, paired end termini, Hfq binding site) would not be applicable as an anti-biofilm molecule and will therefore not be studied further.

The activity of the sense stem loop stem sequence was tested in another *E. coli* strain, TG1. In this strain, a similar effect on biofilm formation could be observed as in *E. coli* DH5α when the complete *csrB* sequence or the sense stem loop stem sequence was expressed. However, the negative effect on biofilm development is smaller than in *E. coli* DH5α. The reason for this is unknown. *E. coli* DH5α carries mutations in its genome. One mutation is in an endonuclease encoding gene, which might cause an increased stability of the RNA molecules in this strain.

The effect of the sense stem loop stem sequence on biofilm development was also studied in *S.* Typhimurium. This is possible because CsrA is widely distributed among eubacterial species (White *et al.*, 1996), and in these species, the activity of this regulatory protein is similarly regulated with sRNAs that mimic its binding site (Babitzke & Romeo, 2007; Figueroa-Bossi *et al.*, 2014; Lapouge *et al.*, 2008; Romeo *et al.*, 2013). *S.* Typhimurium was tested first because in this bacterium, the sequences of *csrA* and *csrB* show strong homology to their *E. coli* counterparts. Moreover, although CsrA predominantly affects virulence in *S.* Typhimurium, the Csr-system was nevertheless shown to be involved in biofilm development (Teplitski *et al.*, 2006). However, nor expressing the complete *csrB* sequence, nor expressing the sense stem loop step sequence has an effect on biofilm development in *S.* Typhimurium in our tests. Therefore, the biofilm effect of the sense stem loop stem sequence is assumed to be species-specific. As *Salmonella* is closely related to *E. coli* and no effect of the sequence is observed in this species, no effect in other species is expected. The reason why no effect on biofilm formation of *S.* Typhimurium in the presence of the sense stem loop stem sequence is observed is unclear. It has already been described that some mutants of genes of the CsrA based regulatory network have a different effect in *S.* Typhimurium and *E. coli* (Teplitski et al., 2006). However, it can also be a matter of concentration as the sequence cannot be induced with IPTG in *S.* Typhimurium because this species lacks a *lac* operon. Therefore, the sequences are continuously expressed in this bacterium, possibly leading to lower expression levels.

CHAPTER 5

PNAs as biofilm inhibitors

PNAs to mimic the effect of plasmid-expressed sequences on biofilm development

In previous chapters, we identified and characterized the sense stem loop stem sequence, which reduces the biofilm forming ability of *E. coli*. However, to be able to use this sequence as a biofilm inhibitor, it needs to be made synthetically so it can be added to the growth medium of the bacteria. In this chapter, a peptide nucleic acid (PNA) sequence based on the sense stem loop stem sequence was tested. The PNA was coupled to a cell penetrating peptide (CPP) to facilitate its uptake. Biofilm tests indicated that this PNA reduced biofilm development of *E. coli* DH5α in a sequence-specific manner, whereas no effect could be observed for *S.* Typhimurium SL1344. However, the control PNA has a negative effect on biofilm formation as well, suggesting that there is also a non-sequence-specific effect of the PNA or the coupled cell penetrating peptide. Although the effect on biofilm formation is similar between cells expressing the sense stem loop stem sequence from a plasmid and cells grown in the presence of the PNA with the same sequence, similar gene expression patterns could not be detected.

5.1. Introduction

Previously, expression of the sense stem loop stem sequence was shown to have an interesting biofilm reducing effect. However, to be able to be applied as a biofilm inhibitor, a synthetic analog of this sequence needs to be developed. These molecules can then be added to the growth medium so they can be taken up by the bacterial cells. Once the PNA has entered the cell, it can interact with the same cellular targets as the plasmid-expressed sequence. Because synthetic DNA or RNA sequences are prone to degradation by nucleases, chemically modified nucleic acid analogs need to be used. Different types of nucleic acid analogs (locked nucleic acids (LNA), peptide nucleic acids (PNA), phosphorodiamidate morpholino oligomers (PMO)) are available. Because these modified nucleic acids are not easily taken up through the low permeable lipopolysaccharide outer membrane of Gram-negative bacteria (Good *et al.*, 2000; Nekhotiaeva *et al.*, 2003), different delivery strategies have been developed to facilitate their cellular uptake. One of these strategies comprises the addition of a cell-penetrating peptide (CPP) to the PNA.

Here, $(KFF)_3K$-coupled PNAs will be used. PNAs are nucleic acid analogs in which the sugar-phosphate backbone is replaced by 2-N-aminoethylglycine units (see **Figure 5.1**). These neutrally charged molecules are more stable against nucleases and proteases than DNA, RNA or proteins and form strong complexes with complementary strands of RNA and DNA (Hatamoto *et al.*, 2009). Therefore, they were proven to be ideal for antisense and antigene applications. However, here, these PNAs will be used as a decoy to mimic the RNA partners of a specific RNA-binding protein, CsrA. The use of PNAs to interact with RNA-binding proteins has not been reported. The $(KFF)_3K$-peptide, which is coupled to the PNA with an ethyleneglycol linker, was the cell-penetrating peptide of choice because $(KFF)_3K$-coupled PNAs have already been shown to be good antimicrobials. Numerous studies show killing of bacteria in the presence of PNAs of this kind targeting essential genes (Alajlouni & Seleem, 2013; Mondhe *et al.*, 2014; Soofi & Seleem, 2012; Tan *et al.*, 2005). Moreover, $(KFF)_3K$-coupled PNAs targeting the essential *acpP* gene are also able reduce the bacterial load and prevent fatal infection in a mouse model with peritonitis (Tan *et al.*, 2005). Studies show that $(KFF)_3K$-coupled antimicrobial PNAs are taken up through permeabilization of the membrane (Eriksson *et al.*, 2002). Additionally, there is a lack of efflux of the CPP-coupled PNA from the cell which results in accumulation in the cell (Nikravesh *et al.*, 2007), making them interesting for antibacterial and also for antibiofilm applications. The mechanism of cell wall penetration by this protein is still under exploration, but might involve the permeabilization of the cell wall (Eriksson *et al.*, 2002).

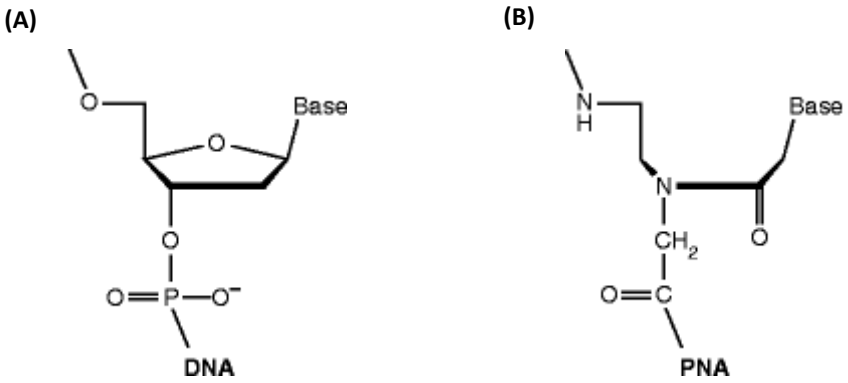


Figure 5.1 Chemical structure of (A) DNA and (B) PNA.

In this chapter, a CPP-coupled PNA based on the sense stem loop stem sequence, which was identified in **Chapter 3** and characterized in **Chapter 4**, will be tested for its ability to reduce biofilm formation when added to a bacterial culture. Additionally, it will be examined whether the mode of action of the PNA is similar to the mode of action of the plasmid-expressed sense stem loop sequence, on which the sequence of the PNA is based.

5.2. Materials and methods

5.2.1 Bacterial strains and growth conditions

Bacteria were grown as described in **section 3.2.1**. The strains that were used in this chapter are listed in **Table 5.1**.

Table 5.1 Bacterial strains

Strain name	Description	Reference
***E. coli* DH5α**	F$^-$ϕ80Δl*acZM15*Δ(l*acZYAargF*)U169 *deoP recA1endA1 hsdR17* (rk$^-$ mk$^-$)	Gibco BRL
***S.* Typhimurium SL1344**	*xyl, hisG, rpsL*; virulent; Sm^R	Hoiseth & Stocker, 1981

5.2.2 Peptide Nucleic Acids (PNAs)

PNAs were synthesized, purified and coupled to the cell penetrating peptide ($(KFF)_3K$) by IDT, (Integrated DNA Technologies, Belgium). They were dissolved in sterile distilled water in a stock concentration of 100 µM. These PNA stocks were consequently divided in aliquots and stored at -20 °C to minimize the detrimental effect of repeated freeze-thaw cycles. The PNAs used in this chapter are listed in **Table 5.2**.

Table 5.2 PNAs. The sequence is given from N-terminus to C-terminus. The N-terminus corresponds to the 3' end of a DNA or RNA sequence (Matsudaira & Coull, 1995).

PNA	Sequence	Based on
PNA1	KFFKFFKFFK-O[1]-GTGTAGGAACAT	Sense stem loop stem (pCMPG10913)
PNA2	KFFKFFKFFK-O[1]-GTGTATTAACAT	Mutated sense stem loop stem (pCMPG10932)

[1] ethyleneglycol linker

5.2.3 Biofilm and growth assay

Biofilms were grown as described in **section 3.2.6**. To test the effect of the PNAs on biofilm development, they were added to the medium (1/100) at the appropriate concentrations. For the biofilms that were grown for 48 hours, the PNAs were again added to the refreshed medium after 24 hours of incubation. The results are based on 3 technical repeats.

To test the effect of the PNA on growth, the OD_{595} of the medium in which the pegs were submerged, was measured in a microplate reader (Synergy MX microtiter plate reader, Biotek Instruments, Inc.).

5.2.4 RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated as described in **section 3.2.3**. cDNA was made as described in **section 3.2.5**. The primers used for qRT-PCR analysis are listed in **Table 3.5**.

5.3. Results

5.3.1 Effect of PNAs on biofilm development

The effect of the PNA based on the sense stem loop stem sequence coupled to a $(KFF)_3K$ cell penetrating peptide (PNA1; see **Table 5.2**), on the biofilm forming capacity of wild type *E. coli* DH5α cells was tested in the Calgary Biofilm device by adding the PNA to the growth medium of the cells in different concentrations. Additionally, a control PNA (PNA2; see **Table 5.2**) was included in the biofilm test. The sequence of this PNA is based on one of the mutated sense stem loop stem sequences (pCMPG10932), which was shown to have no effect on biofilm development when expressed from a plasmid (see **Figure 4.2**). The results of the biofilm assay with the PNAs is shown in **Figure 5.2**.

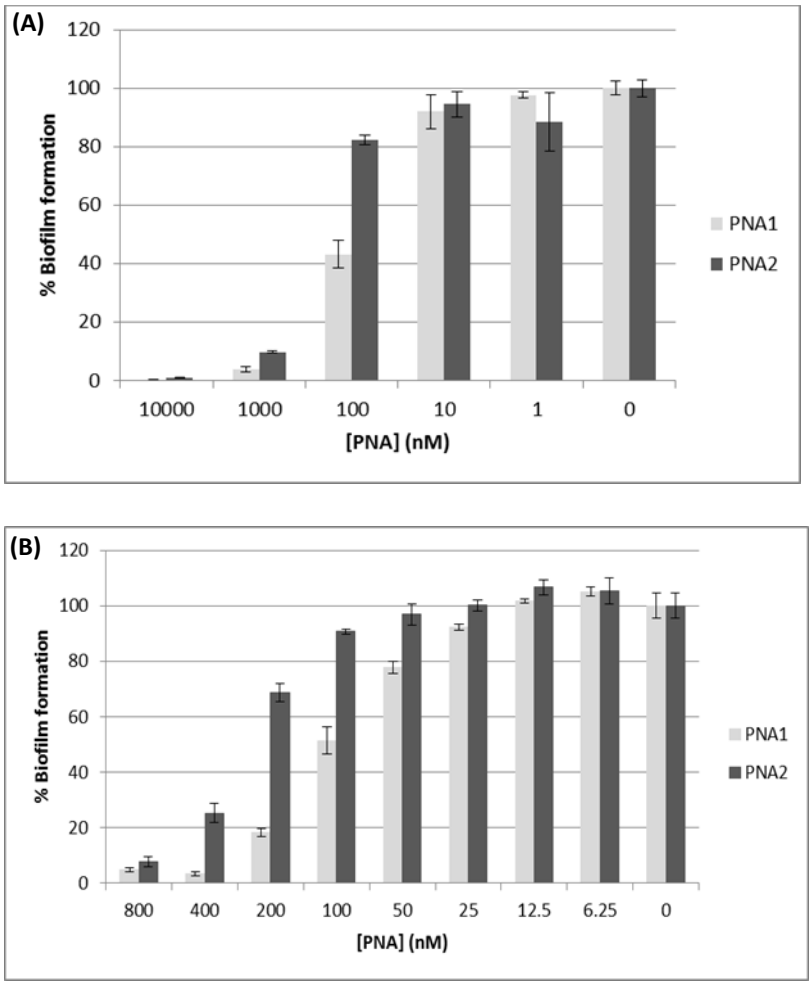


Figure 5.2 Biofilms formed by *E. coli* DH5α strains in the presence of PNA1 and PNA2 at different concentrations after 48 hours of growth (A) for a large concentration gradient and (B) for a concentration gradient that was narrowed down. Error bars represent standard deviations. The amount of biofilm formed by strains when no PNA was added to the medium is set at 100 %.

From **Figure 5.2** it is clear that biofilm formation by *E. coli* when grown in 1/20 TSB is approximately 50 % reduced in the presence of 100 nM of the PNA that is based on the sense stem loop stem sequence (PNA1), while for the PNA that is based on a mutated form of the sense stem loop stem sequence (PNA2), a higher concentration needs to be added before the same reduction in biofilm formation can be observed. These data suggest that PNA1 reduces biofilm formation in a sequence-specific manner. However, as biofilm development is also reduced in the presence of the control PNA (PNA2), although at higher concentrations, this indicates that the PNAs also have a non-sequence-

specific reducing effect on biofilm formation. Measurements of the OD_{595} of the medium surrounding the pegs, representive for the planktonic growth of residual cells in the medium that have not attached to the pegs, demonstrate that although biofilm formation is reduced, planktonic growth is not abolished in the presence of the PNAs, at none of the concentrations tested (see **Figure 5.3**).

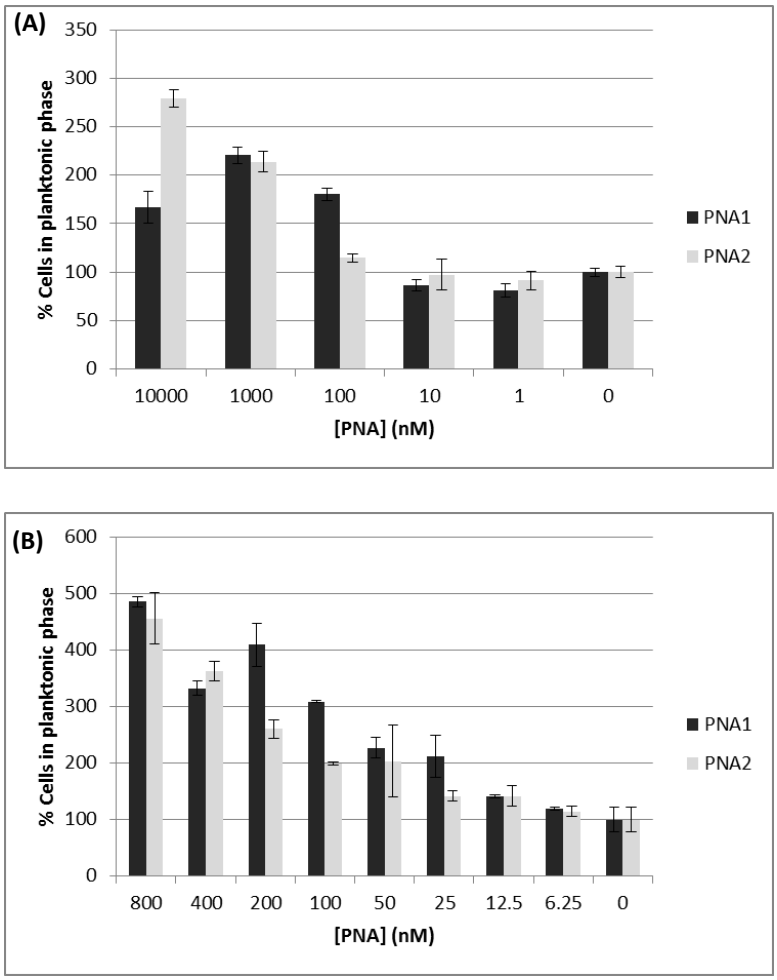


Figure 5.3 Residual *E. coli* DH5α cells present in planktonic phase, which is the medium in which the pegs, on which the biofilm has formed, were submerged in the presence of PNA1 and PNA2 at different concentrations after 48 hours of growth (A) for a large concentration gradient and (B) for a concentration gradient that was narrowed down. Error bars represent standard deviations. The growth of strains when no PNA was added to the medium is set at 100 %.

5.3.2 Effect of PNAs on biofilm formation of other bacteria and in other growth media

To test the species-specificity and the condition-dependency of the PNA, the biofilm forming ability of *S.* Typhimurium SL1344 grown in 1/20 TSB and of *E. coli* DH5α grown in LB at 37 °C in the presence of the PNAs was tested. The results are shown in **Figure 5.4** and **Figure 5.5**.

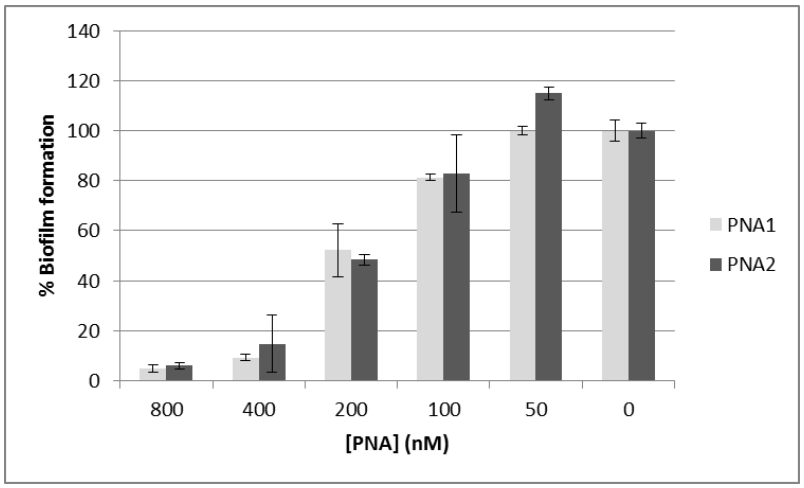


Figure 5.4 Biofilms formed by *S.* Typhimurium SL1344 in the presence of PNAs at different concentrations after 48 hours of growth. Error bars represent standard deviations. The amount of biofilm formed by strains when no PNA was added to the medium is set at 100 %.

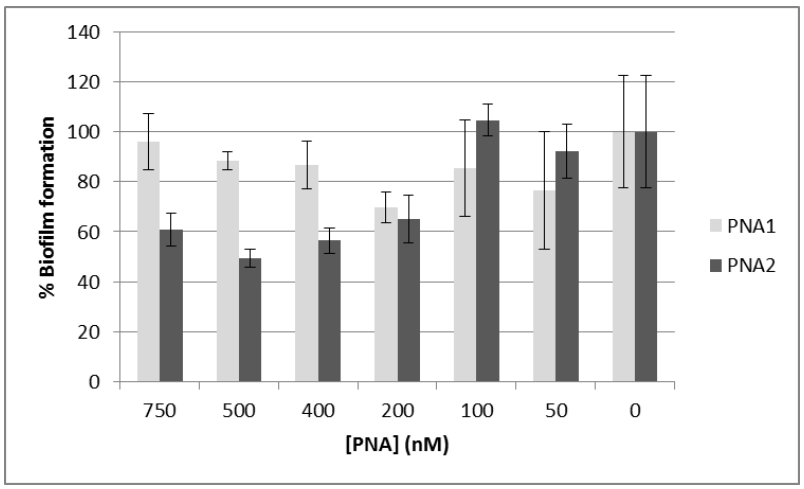


Figure 5.5 Biofilms formed by *E. coli* DH5α in the presence of PNAs at different concentrations after 24 hours of growth. The strains were grown in the Calgary Biofilm Device in LB at 37 °C. Error bars represent standard deviations. The amount of biofilm formed by strains when no PNA was added to the medium is set at 100 %.

The results in **Figure 5.4** show that the amount of biofilm that is formed by *S*. Typhimurium when grown in 1/20 TSB at 25°C is not different in the presence of PNA1 or PNA2, suggesting that PNA1 has no sequence-specific effect on biofilm formation in this bacterium. This corresponds to the results in **section 4.3.3** that showed that the sense stem loop stem sequence did not affect the biofilm forming capacity of *S.* Typhimurium SL1344. However, similar to what was observed for *E. coli*, the PNAs have a non-sequence-specific reducing effect on the biofilm forming ability of *S.* Typhimurium. This effect becomes stronger with increasing PNA concentrations.

The results for *E. coli* DH5α grown in LB at 37°C (see **Figure 5.5**) show that in the presence of PNA2, biofilm formation is reduced at a concentration of 200, 400, 500 and 750 nM, when compared to the amount of biofilm that is formed in the absence of the PNAs. However, in the presence of PNA1, this reduction in the amount of biofilm was not observed. As PNA2 is assumed to be the control, these data suggest that, contrary to the results for the strains grown in 1/20 TSB, PNA1 has a sequence-specific positive effect on biofilm development of *E. coli* when grown in LB at 37 °C. This is in agreement with the condition-dependent effect of regulating CsrA activity (see **section 3.3.4**). Remarkably, the non-sequence-specific reduction in biofilm formation in the presence of PNA2 is less severe compared to the effect on cells grown in 1/20 TSB.

5.3.3 CsrA target gene expression analysis

Adding the PNA that is based on the sense stem loop stem sequence (PNA1) to the growth medium of *E. coli* DH5α cells has a negative effect on biofilm development, similar to what was observed when the same RNA sequence was expressed from a plasmid inside the cell. Consequently, it was tested whether PNA1 and the plasmid-expressed sense stem loop stem sequence have the same mode of action. Therefore, the expression level of the previously tested CsrA targets was determined for cells grown in the presence of the PNAs, as the sense stem loop stem sequence was shown to affect the expression of these genes. Hereto, RNA samples were taken from strains grown under shaking conditions in 1/20 TSB at 25 °C in early exponential phase (OD 0.1), both in the presence of 100 nM of PNA1 or PNA2 or in the absence of PNAs. These are the same conditions as for the experiments described in **section 4.3.2.** A concentration of 100 nM was chosen because in the presence of 100 nM of PNA1, biofilm formation of *E. coli* DH5α was 50 % reduced, while in the presence of 100 nM of PNA2, almost no reduction in the biofilm forming capacity of *E. coli* DH5α could be detected. Therefore, we expect to observe predominantly the sequence-specific effect of

PNA1.

The results of the qRT-PCR analysis, shown in **Figure 5.6**, demonstrate that the expression of the CsrA targets *ycdT*, *ydeH*, *glgC* and *pgaA* was not upregulated and the expression of *fliA* was not downregulated in the presence of PNA1 when compared to the expression levels of cells grown in the absence of PNAs, unlike when the sense stem loop stem sequence was expressed form the plasmid. On the contrary, *ycdT* expression was slightly downregulated in the presence of PNA1. In the presence of PNA2, no significant differences in the expression of these genes could be observed if compared to the expression of the genes in the absence of PNA.

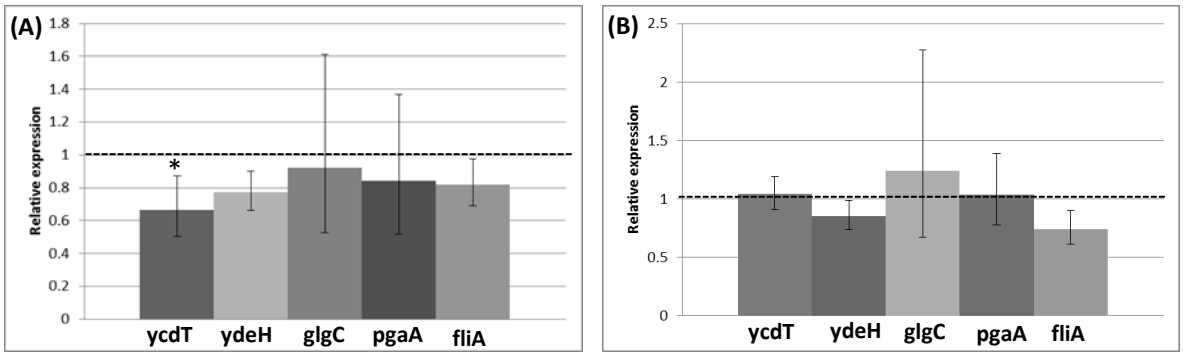


Figure 5.6 Relative expression levels of a selected number of CsrA targets in *E. coli* DH5α cells grown in 1/20 TSB at 25 °C in the presence of 100 nM (A) of the PNA based on the sense stem loop stem sequence (PNA1) and (B) of the control PNA (PNA2). Expression levels were compared to the expression levels of *E. coli* grown in the absence of PNA. Error bars represent 95 % confidence intervals. A star indicates that the C_T values are significantly different for both strains ($p < 0.05$). The dotted line indicates a relative expression level of 1. Relative expression levels > 1 indicate increased expression, relative expression levels <1 indicate reduced expression compared to when no PNA is added.

As previous experiments showed that larger expression differences could be observed when strains were grown in LB, the expression levels of the same genes were measured for cells grown in LB at 25°C in the presence of 100 nM of PNA1. Samples were again taken in exponential phase (OD 1). The results are shown in **Figure 5.7**.

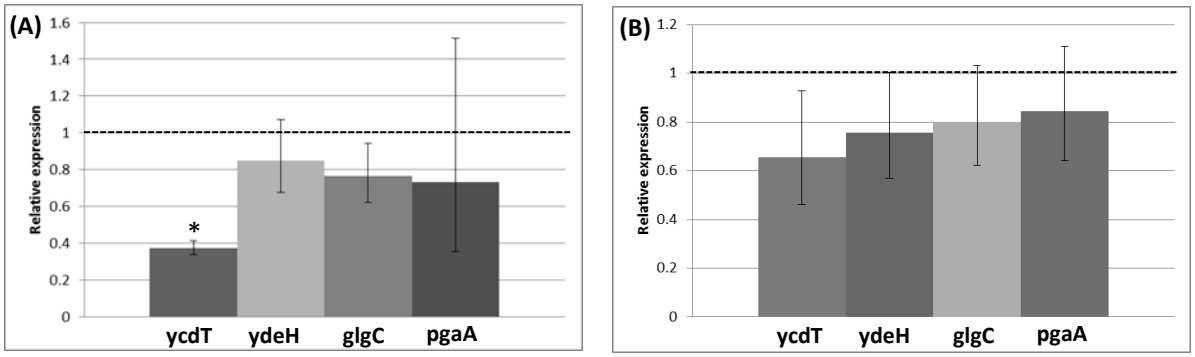


Figure 5.7 Relative expression levels of a selected number of CsrA targets in *E. coli* DH5α cells grown in LB at 25 °C in the presence of 100 nM (A) of the PNA based on the sense stem loop stem sequence (PNA1) and (B) of the control PNA (PNA2). Expression levels were compared to the expression levels of *E. coli* grown in the absence of PNA. Error bars represent 95 % confidence intervals. A star indicates that the C_T values are significantly different for both strains ($p < 0.05$). The dotted line indicates a relative expression level of 1. Relative expression levels > 1 indicate increased expression, relative expression levels <1 indicate reduced expression compared to when no PNA is added.

The results in **Figure 5.7** show that when the cells are grown in LB in the presence of 100 nM PNA1, *ycdT* expression is significantly reduced when compared to cells grown in the absence of PNA, the expression of none of the other genes tested differed significantly. Although the expression of *ycdT* was also predominantly affected when the sense stem loop stem was expressed, in the presence of PNA1, *ycdT* expression is significantly downregulated, while its expression was significantly upregulated when the sense stem loop stem sequence was expressed. These data suggest that although the PNA and the sequence on which it is based both reduce biofilm development, it could not be confirmed yet that their mode of action is similar.

5.4. Discussion

In a previous chapter, the sense stem loop stem sequence was identified as an interesting sequence to reduce CsrA activity and thereby disturb biofilm formation of DH5α grown in 1/20 TSB at 25 °C. Consequently, this sequence was converted into a synthetic PNA which can be added to the growth medium from where it can be taken up by the bacteria. This is facilitated by a cell penetrating

peptide coupled to the PNA. The PNA based on the sense stem loop stem (PNA1) reduces biofilm formation of *E. coli* in a sequence-specific manner. Biofilm formation was 50 % reduced at a concentration of 100 nM, which is significantly lower than the IC_{50} values that were measured for other antimicrobial antisense PNAs targeting essential genes in this bacterium (Bai & Luo, 2012). Additionally, a sequence-specific effect of the PNA on biofilm development was not observed in *S.* Typhimurium, confirming earlier results of experiments with plasmid-expressed sequences.

As biofilm formation is also reduced in the presence of the control PNA, both in *E. coli* and in *S.* Typhimurium, the data suggest that also non-sequence-specific effects play a role in the negative effect of PNA1 on biofilm development. This might be caused by the CPP that is attached to the PNA sequences. Although the mechanism of cell wall penetration by the CPP is still controversial, it is thought to involve the permeabilization of the cell membrane by the formation of a pore by cationic and hydrophilic residues (Bai & Luo, 2012; Herce *et al.*, 2009). Since biofilm formation is highly interconnected with outer membrane remodeling (reviewed in Van Puyvelde et al., 2013), destabilizing the membrane might affect the ability to form biofilms. Additionally, as the CPP used is shown to have antimicrobial activity itself at high concentrations (Patenge *et al.*, 2013), this might also affect the amount of biofilm that is formed. To circumvent the toxicity of the $(KFF)_3K$ CPP, other approaches can be used to facilitate the uptake of nucleic acid analogs by bacteria (Bai & Luo, 2012). Firstly, there are other CPPs available, such as $(RXR)_4XB$ (X is 6-aminohexanoic acid and B is β-alanine), which is more efficiently taken up in some bacteria and can therefore be used at lower concentrations (Bai *et al.*, 2012). Besides synthetic peptides, like the two already mentioned, there are also naturally occurring CPPs, such as the HIV-1 Tat-derived arginine rich recombinant peptide which destabilizes lipid bilayers and forms transient pores (Gupta *et al.*, 2005; Herce *et al.*, 2009). This peptide shows lower toxicity compared to the synthetic CPPs (Patenge *et al.*, 2013). Secondly, the nucleic acid analogs can be delivered in liposomes or with nano-materials. The latter have been developed recently and are under study as a delivery tool. However, for therapeutic purposes, CPPs still outperform other delivery strategies (Bai & Luo, 2012).

Although the sequence-specific effect of the PNA on biofilm development is similar compared to its plasmid-expressed counterpart, no similar effect on the expression of the CsrA target genes could be observed. It might be that the PNAs do act on CsrA, but that gene expression differences are smaller because of concentration differences. The number of sequences expressed from the plasmid can indeed be higher than the number of PNAs that is taken up. This hypothesis is strengthened by the fact that also the effect on biofilm development is smaller when grown in the presence of 100 nM of PNA1 than when the sense stem loop stem sequence is induced with 1 mM of IPTG from the plasmid.

Therefore, it might be interesting to take samples and analyze gene expression in the presence of higher concentrations of PNA, although the uptake through the membrane is probably not infinite.

Furthermore, the application of the PNA is possibly prone to optimalization, especially with regard to the efficiency. Possibly, the CPP reduces the activity of the sequence because of sterical hindrance, which might be solved by including a more flexible linker between the analog and the CPP, although the polyethylenglycol linker that was used here is the most common one applied for antimicrobial PNAs (Bai & Luo, 2012). Additionally, it might also be that PNAs are not the ideal nucleic acid analogs for this purpose. PNAs have been extensively tested for their use as an antimicrobial, targeting essential genes by antisense molecules, which causes cell death (Alajlouni & Seleem, 2013; Ghosal & Nielsen, 2012; Mondhe *et al.*, 2014; Patenge *et al.*, 2013; Soofi & Seleem, 2012; Tan *et al.*, 2005). Alternatively, PNAs were used to target bacterial resistance genes, causing restoration of susceptibility (Jeon & Zhang, 2009; Wang *et al.*, 2010; White *et al.*, 1997) (reviewed in Bai and Luo, 2012). For these applications, it is necessary that the PNAs base pair near the translation start of the mRNA of interest. Therefore, they need to form strong complexes with complementary DNA or RNA. Indeed, PNAs have no charge, preventing repulsion between the PNA and the interacting DNA and RNA. However, the sense stem loop stem sequences, and consequently PNA1 developed here, is designed to function as a protein binding sequence and does not need to bind complementary DNA or RNA. Perhaps the natural negative charge of RNA is necessary for protein interaction. Therefore, negatively charged nucleic acid analogs, like locked nucleic acids (LNAs), might be more suitable. LNAs are nucleic acid analogs in which the ribose moiety is modified with a bond connecting the 2' oxygen and 4' carbon atom, making it more resistant against nucleases, but the phosphodiester bond is still present. However, previous attempts to conjugate a CPP to negatively charged analogs did not result in a level of delivery into cells sufficient for biological activity, hampering their application (Bai & Luo, 2012). Alternatively, it might be useful to test chimeric DNA/PNA or chimeric RNA/PNA molecules. Double stranded PNA-DNA-PNA or DNA/PNA-DNA-PNA chimeras were shown to be efficient decoy molecules to interact with transcription factors in eukaryotes (Borgatti *et al.*, 2003). Although transcription factors are DNA-binding proteins and not RNA-binding proteins, the results might be applicable to both types of nucleic acid binding proteins. Together with the fact that these chimeras are more resistant to nucleases than DNA/DNA hybrids, this suggests that chimeras are a good alternative for our application. Possibly, a chimera of PNA and RNA is even better. This type of chimeras have already been used in eukaryotes in an antisense application (Potenza *et al.*, 2008).

CHAPTER 6

THE MIRROR SEQUENCE

Another sequence that reduces the ability to form biofilms

In this chapter, focus is on the mirror stem loop stem sequence, a sequence that was unexpectedly shown to negatively affect biofilm formation and growth in *E. coli* DH5α. To unravel the mechanism underlying this growth and biofilm defect, a whole transcriptome analysis (RNA-seq) was done. This revealed that curli-specific genes and motility genes were downregulated and genes necessary to grow in conditions of low pH were upregulated in the presence of the mirror stem loop stem sequence. As the sequence might be active by competing for regulatory protein binding, common regulators of a number of differentially expressed genes were identified. This led to the observation that a reduced activity of H-NS could explain a large number of the gene expression differences. Alternatively, by comparing the recognition sequence of the identified regulators with the characteristics of the mirror sequence, Lrp could be assigned as another candidate target of the mirror stem loop stem sequence. Finally, the effect of expressing the mirror stem loop stem sequence was evaluated in other bacterial strains and species. In *E. coli* TG1 and *S.* Typhimurium, expressing the mirror stem loop stem sequence negatively affects biofilm development, similarly to what was observed for *E. coli* DH5α. However, expressing this sequence in *S.* Typhimurium has an even stronger effect on the planktonic growth, which might contribute to the biofilm defect of these bacteria.

6.1. Introduction

In this chapter, the focus will be on the second sequence that was shown to negatively affect biofilm formation in **Chapter 3**, the mirror stem loop stem sequence. Since this sequence was included as a negative control in the search for sequences that interfere with the CsrA-based regulatory network, the observed effect on biofilm formation and growth was unexpected. Here, we will try to unravel the mechanism by which the mirror stem loop stem sequence affects biofilm formation and growth. Additionally, possible targets of the mirror stem loop stem sequence will be identified. Subsequently the effect of expressing this sequence will be tested in *E. coli* TG1 and in *S.* Typimurium.

6.2. Materials and methods

6.2.1 Bacterial strains and growth conditions

Bacteria were grown as described in **section 3.2.1**. The specific strains and plasmids used in this chapter are listed in **Table 6.1** and **6.2**.

Table 6.1 Bacterial strains

Strain name	Description	Reference
***E. coli* DH5α**	F$^-$ϕ80Δl*acZM15*Δ(l*acZYAargF*)U169 *deoP recA1endA1 hsdR17* (rk$^-$ mk$^-$)	Gibco BRL
***E. coli* Top10**	F′ {*lacIq* Tn10(TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80*lacZΔM15 ΔlacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (*StrR*) *endA1 nupG*	Invitrogen
***E. coli* TG1**	F' *Δlac pro supE thi hsdD5 traD36 proAB+, lacIq lacZΔM15*	Amersham Pharmacia Biotech
***S.* Typhimurium SL1344**	*xyl, hisG, rpsL*; virulent; Sm^R	Hoiseth & Stocker, 1981

Table 6.2 Plasmids

Plasmid name	Description	Reference
pJV853-1	Anti-*micA* expression construct based on pZE12-luc. P_{LlacO} promoter, Ap^R	Gift from J. Vogel, published in (Kint *et al.*, 2010)
pCMPG10901	pJV853-1 based plasmid, expressing mirror stem loop stem, constructed with PRO-7172 and PRO-7336, *PstI* restriction site	Chapter 3
pCMPG10907	pJV853-1 based plasmid, expressing random sequence, constructed with PRO-7172 and PRO-7573, *PstI* restriction site	Chapter 3
pCMPG10908	pJV853-1 based control plasmid, no sequence incorporated, constructed with PRO-7171 and PRO-7172, *PstI* restriction site	Chapter 3
pCMPG10912	pJV853-1 based plasmid, expressing mirror stem loop, constructed with PRO-8149 and S&P-00414, *EcoRI* restriction site	This work
pCMPG10924	pJV853-1 based plasmid, expressing mutated mirror stem loop stem, constructed with S&P-00550 and PRO-7172 (AGGA -> ATTA), *PstI* restriction site	This work
pCMPG10925	pJV853-1 based plasmid, expressing mutated mirror stem loop stem, constructed with S&P-00551 and PRO-7172 (AGGA -> ATTA), *PstI* restriction site	This work
pCMPG10926	pJV853-1 based plasmid, expressing mutated mirror stem loop stem, constructed with S&P-00552 and PRO-7172 (only AGGA and restriction site conserved), *PstI* restriction site	This work
pCMPG10927	pJV853-1 based plasmid, expressing mutated mirror stem loop stem (mutated loop), constructed with S&P-00553 and PRO-7172, *PstI* restriction site	This work
pCMPG10928	pJV853-1 based plasmid, expressing mutated mirror stem loop stem (mutated loop), constructed with S&P-00554 and PRO-7172, *PstI* restriction site	This work

6.2.2 Construction of plasmids

Plasmids were constructed as described in **section 3.2.2**. All the primers that were used to construct the different plasmids in this chapter are listed in **Table 6.3**.

Table 6.3 Primers

Primer	Sequence	Purpose	
PRO-7172	ATCTGCAGGGCTCAGTCGAAAGACTG	Rv	Every pJV853-1 based plasmid with *PstI* restriction site
PRO-8349	ATGAATTCGGCTCAGTCGAAAGACTG	Rv	Every pJV853-1 based plasmid with *EcoRI* restriction site
S&P-00414	ATGAATTCCATGTTCCTACAGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10912
S&P-00550	ATCTGCAGCATGTTAATACAGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10924
S&P-00551	ATCTGCAGCATGTTGGTACAGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10925
S&P-00552	ATCTGCAGCCCCTCCTGGGGGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10926
S&P-00553	ATCTGCAGCTCTTAGCTACTGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10927
S&P-00554	ATCTGCAGTGAATGCCTACTGTGCTCAGTATCTTGTTATCCG	Fw	pCMPG10928

6.2.3 RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated as described in **section 3.2.3**. cDNA was made as described in **section 3.2.5**. The primers used for qRT-PCR analysis are listed in **Table 3.5**. The qRT-PCR primers that were used for the first time in this chapter are listed in **Table 6.4**.

Table 6.4 qRT-PCR primers

Primer	Sequence	Purpose		Reference
S&P-00860	GCGTATCGGCGCGTCTA	Fw	csgD	This work
S&P-00861	AACCGTATTTTCGCTGATGAACA	Rv	csgD	This work
S&P-01144	CGGCAGGGAGGCTCAAA	Fw	csgB	This work
S&P-01145	TTGCCCGGTTGCTACTACCTT	Rv	csgB	This work
S&P-01146	CCTGGACACGCTGCTTGAT	Fw	fliZ	This work
S&P-01147	GCCGCCCATGATTTTTGTT	Rv	fliZ	This work
S&P-01148	TCGATCTGGTCCAGGAAGGT	Fw	motB	This work
S&P-01149	TCTAAACATCGGGCGATTCTG	Rv	motB	This work
S&P-01150	CTGACCCCATGCCGAACT	Fw	rrfA	This work
S&P-01151	TGAGGAGACCCCACACTACCA	Rv	rrfA	This work
S&P-01152	CATCTCTCCGTAAAGCGTTTATTTTT	Fw	hdeB	This work
S&P-01153	TTGTGCGTTCACCAGTGACA	Rv	hdeB	This work
S&P-01154	CCTGCGCCGAAAAATGG	Fw	gadB	This work
S&P-01155	GCCTCGGAAGAACCAATGG	Rv	gadB	This work

6.2.4 RNA sequencing analysis

Samples for RNA-sequencing were taken from cultures grown shaking in 1/20 TSB at 25°C at an OD_{595} of 0.1 (early exponential phase). After RNA isolation (see **section 3.2.3**), the RNA samples (20 µg) were sent to Beijing Genomics Institute (BGI) for removal of ribosomal RNA, library construction (fragmentation, first and second strand cDNA synthesis, adapter ligation and second strand cDNA degradation and PCR amplification) and paired end sequencing on an Illumina HiSeq™2000 platform (100 bp read length). Afterwards, the raw reads were filtered by removing adaptor sequences, contamination and low-quality reads, all according to the standard protocols used by BGI. Subsequently, the sequencing data were analyzed using Bioconductor R packages. Rsubread was used to map the sequences on the *E. coli* genome (NC_000913), edgeR was used to normalize the data, conditioning on the total count for that gene. Finally, differential expression is assessed for each gene using an exact test analogous to Fisher's exact test, but adapted for overdispersed data (Robinson *et al.*, 2010). The results are based on three biological repeats.

6.2.5 Gene ontology analysis

A gene ontology analysis (Ashburner et al., 2000) was done by entering the gene list in the Enrichment Analysis Tool on the gene ontology website (http://geneontology.org/).

6.2.6 Biofilm and growth assay

Biofilms were grown in the Calgary Biofilm Device as described in **section 3.2.6**. Additionally, in this chapter, biofilm formation was also evaluated by CFU counts. Hereto, an overnight culture of cells was diluted 1/100 in 10 ml 1/20 TSB. Consequently, cells were grown non-shaking in a small petri dish at 25 °C. After the appropriate incubation time, 1 ml of the planktonic phase, which is the medium above the surface attached cells, was plated in serial dilutions. Afterwards, the remaining medium was poured off and the surface attached cells were scraped off and suspended in 1 ml of PBS. After pulling it through a needle (25G, Becton Dickinson) to break up aggregated cells, the suspension was plated in serial dilutions as well. The results are based on 3 technical repeats.

6.2.7 Swarming assay

For the swarming assay, 2 µl of an overnight culture was spotted on a LB plate containing 0.6 % agar and 0.5 % glucose. The plates were incubated at 30 °C for 24 hours and were inspected visually for swarming patterns.

6.3. Results

6.3.1 CFU counts of cells in planktonic phase and biofilm phase

The results in **Chapter 3** already showed that biofilm formation of strains expressing the mirror stem loop stem sequence is reduced after 24 and 48 hours of growth in 1/20 TSB in the Calgary Biofilm Device and that also growth is negatively affected in the presence of the sequence. To obtain insight into the dynamics of biofilm formation and the ratio of cells in planktonic and in biofilm phase in the presence of the mirror sequence, the CFU of the cells in both phases was determined every 2 hours for 24 hours. The results are shown in **Figure 6.1**. CFU measurements are different from coloring with crystal violet because crystal violet stains both the matrix, the dead and live cells, while with CFU counting only the living cells are measured .

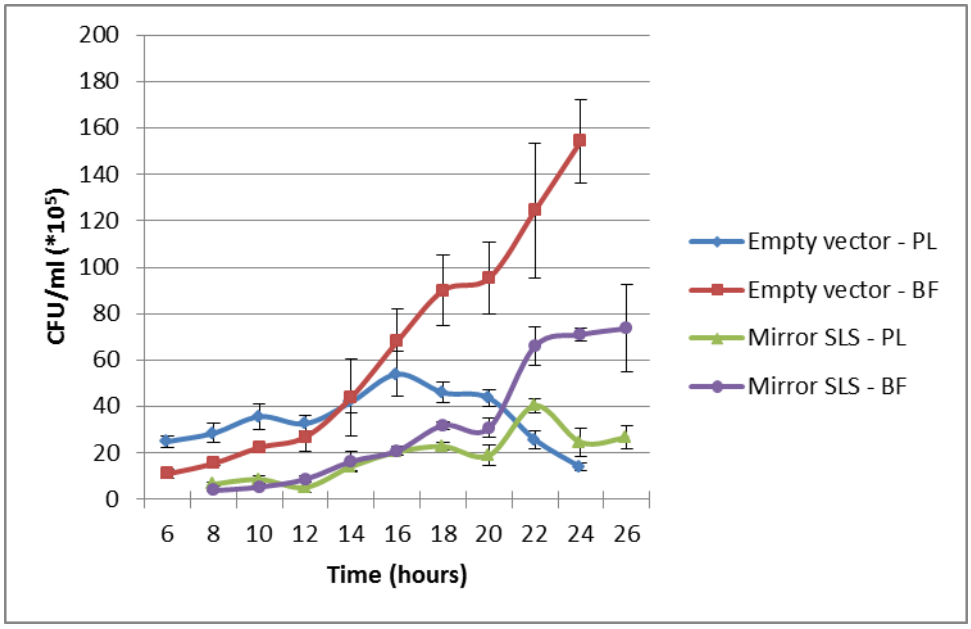


Figure 6.1 CFU count of cells in biofilm phase (BF) and planktonic phase (PL) of *E. coli* DH5α strains with the mirror stem loop stem sequence (pCMPG10901) and strains with the empty vector (pCMPG10908). The strains were grown for 24 hours and every 2 hours both the biofilm phase, attached to the bottom of the petri dish, and the planktonic phase, present in the culture above the biofilm, were plated.

In **Figure 6.1**, it can be observed that both the biofilm and the planktonic growth is delayed for strains expressing the mirror stem loop stem sequence (pCMPG10901) compared to strains with the empty plasmid (pCMPG10908). This leads to a reduced amount of biofilm after 24 hours of incubation, comparable to the measurements in the Calgary Biofilm Device (see **Figure 3.8**). For both strains, the increase in the number of cells over time is higher for cells in biofilm phase than for cells in planktonic phase, suggesting that biofilm growth is preferred under the conditions tested.

6.3.2 Gene expression analysis of CsrA targets

It was unexpected to observe a reduced biofilm formation and growth for strains expressing the mirror stem loop stem sequence, as the sequence was included as a negative control in the search for RNA sequences that affect CsrA activity. Although the mirror stem loop stem sequence (pCMPG10901) does not resemble the CsrA recognition site as good as the sense stem loop stem sequence (pCMPG10905), GGA nucleotides are present in the sequence. Additionally, the sequence

contains nucleotides that can base pair (UGU and ACA), enabling the formation of a stem loop structure. As these are two elements that describe a CsrA binding sequence, it is possible that this mirror sequence is acting just like the sense construct, sequestering CsrA. Therefore, the expression level of the known CsrA targets was measured, similar to the experiments done in **section 3.3.4** and **4.3.2**. The results are shown in **Figure 6.2**.

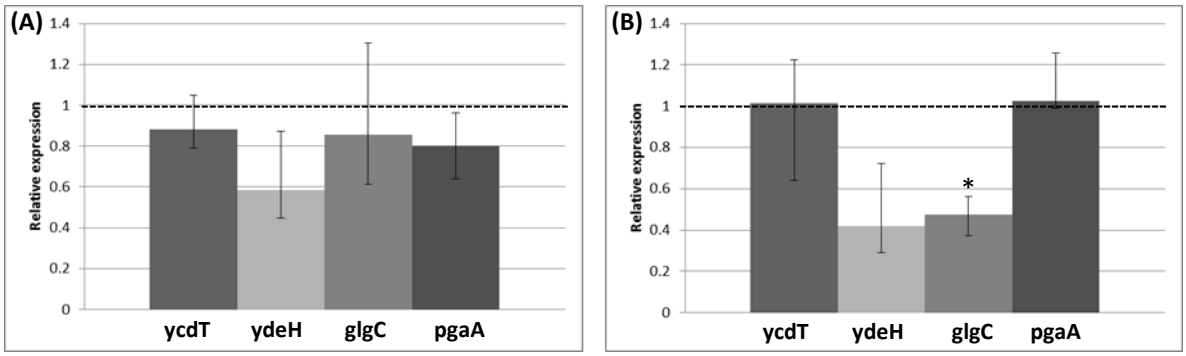


Figure 6.2 Relative expression levels of a selected number of CsrA targets in cells expressing the mirror stem loop stem sequence (pCMPG10901) relative to cells with the empty vector (pCMPG10908). (A) For cells grown in 1/20 TSB at 25°C. **(B)** For cells grown in LB at 25°C. Error bars represent 95% confidence intervals. A star indicates that the C_T values are significantly different for both strains ($p < 0,05$). The dotted line indicates a relative expression level of 1. Relative expression levels > 1 indicate increased expression, relative expression levels <1 indicate reduced expression compared to the control strain.

The results in **Figure 6.2** indicate that the expression of a number of CsrA target genes, which were also tested in **Chapters 3** and **4**, is not upregulated in the presence of the mirror sequence. This would be expected if the sequence was sequestering CsrA. Indeed, sequestering CsrA would mean that less CsrA is present to downregulate the expression of its mRNA targets, leading to an increased expression of these targets. The different gene expression pattern between cells expressing the mirror stem loop stem sequence and cells expressing the sense stem loop stem sequence (see **Figure 4.3** and **4.4**) suggests that both sequences have a different working mechanism and negatively affect biofilms by altering different sets of genes.

6.3.3 RNA-seq analysis

The small gene expression study in previous section showed that the negative effect of the mirror stem loop stem sequence on biofilm development is not caused by reducing CsrA activity. To understand how the sequence does affect biofilm formation and growth, a whole transcriptome analysis (RNA-seq) was done, comparing the gene expression profile of cells expressing the mirror stem loop stem sequence (pCMPG10901) to the expression profile of cells with an empty vector (pCMPG10908). Hereto, RNA samples were taken early in exponential phase (OD 0.1; see **Figure 6.3**) for strains grown shaking in 1/20 TSB at 25 °C. Samples were not taken in biofilm phase, because biofilm formation is severely affected in the presence of the mirror stem loop stem sequence, so for this strain, samples were taken in planktonic phase. To make a good comparison, samples of strains with the empty vector were also taken in planktonic phase. Although this approach will not reveal the gene expression differences in biofilm cells, the genes and processes that are affected in planktonic growth can also be interesting and can also give insight into the reason why biofilm formation is reduced. As the sequence affects the growth, it clearly has a target that is expressed under planktonic conditions. If the target is a global regulator, it might also be responsible for the reduced biofilm formation. Additionally, because the switch from free living to a surface attached mode of growth needs to be prepared in planktonic growth, some specific genes that are necessary for biofilm development are already expressed under planktonic growth, and therefore the effect on these genes can be detected as well (Van Puyvelde, 2014). At an OD_{260} of 0.1, the growth speed between the strains is different, suggesting that gene expression differences caused by the sequence can be observed. At the same time, both strains are in a similar growth phase, so the likelihood that the differentially expressed genes are attributed to growth phase-dependent expression differences are minimized.

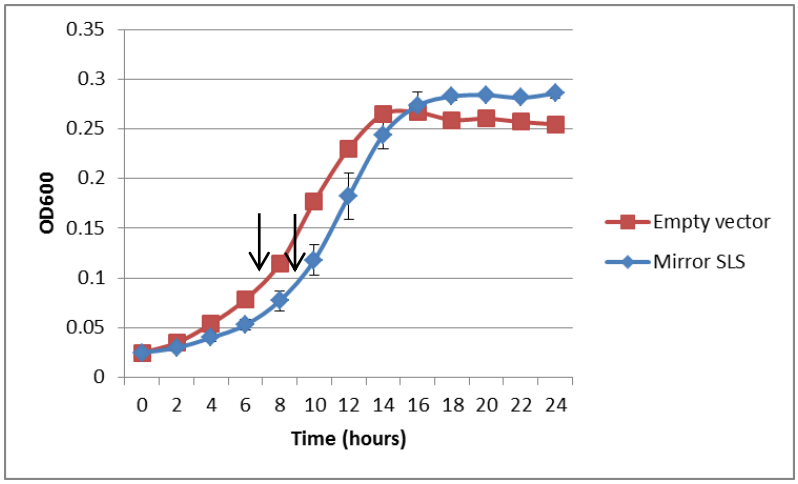


Figure 6.3 Growth curve of strains expressing the mirror stem loop stem sequence (Mirror SLS - pCMPG10901) and cells with the empty vector (pCMPG10908). Bacteria were grown shaking, in flasks, in 1/20 TSB at 25°C. Error bars represent standard deviations. Arrows indicate the time point at which samples were taken.

For the samples expressing the mirror stem loop stem sequence, about 82 to 84 percent of the reads could be mapped on the *E. coli* K12 MG1655 (NC_000913) reference genome, compared to 92 percent for the samples with the empty vector[2]. Some of the unmapped reads can be attributed to sequences expressed from the vector. For example, the mRNA of the *bla* gene responsible for ampicillin resistance, will not be mapped onto the reference genome, but is present as the strain is ampicillin resistant. Based on the mapped reads, fold changes were calculated. Using a log fold change cutoff of (+/-) 1.5 and taking into account the p-value ($p < 0.05$), a list of differentially expressed genes was generated. This list is shown in **Table 6.5**. The expression of 100 genes was downregulated and the expression of 53 genes was upregulated in the presence of the mirror stem loop stem sequence. Subsequently a Gene Ontology enrichment analysis was done to identify which processes are over- or underrepresented within the list of genes that are down- or upregulated in the presence of the mirror stem loop stem sequence (**Table 6.6** and **Table 6.7**, respectively) (Mi *et al.*, 2013).

[2] Bioinformatic analyses of the RNA-seq data were performed by Sandra Van Puyvelde.

Table 6.5 Differentially expressed genes. logFC = log 2 of the fold change (FC), the fold change is the ratio of the expression levels of cells expressing the mirror stem loop stem sequence compared to cells with an empty vector.

Gene name	Function	LogFC	FC	P-Value
csgB	curlin nucleator protein, minor subunit in curli complex	-7.60331	0.005143	1.99E-263
putA	fused DNA-binding transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	-6.13913	0.014189	1.00E-276
csgA	curlin subunit, amyloid curli fibers, cryptic	-6.08044	0.014778	4.27E-169
csgF	curli nucleation outer membrane protein	-3.85546	0.069086	7.49E-133
csgE	curlin secretion specificity factor	-3.67816	0.07812	7.14E-91
tar	methyl-accepting chemotaxis protein II	-3.5884	0.083135	1.78E-67
csgD	csgBAC operon transcriptional regulator	-3.55158	0.085284	4.31E-142
fliA	RNA polymerase, sigma 28 (sigma F) factor	-3.53939	0.086008	4.18E-67
csgC	curli assembly protein	-3.53762	0.086113	3.70E-26
fliD	flagellar filament capping protein	-3.5254	0.086846	1.92E-80
fliC	flagellar filament structural protein (flagellin)	-3.5249	0.086876	3.67E-70
fliS	flagellar protein potentiates polymerization	-3.52313	0.086983	9.51E-65
motA	proton conductor component of flagella motor	-3.49218	0.088869	2.13E-85
csgG	curli production assembly/transport outer membrane lipoprotein	-3.46663	0.090457	1.96E-132
yjbT	putative periplasmic protein	-3.31846	0.100241	5.50E-64
cheR	chemotaxis regulator, protein-glutamate methyltransferase	-3.31504	0.100479	1.88E-58
fliZ	RpoS antagonist; putative regulator of FliA activity	-3.30114	0.101451	3.70E-95
flgC	flagellar component of cell-proximal portion of basal-body rod	-3.28988	0.102246	8.55E-76
tap	methyl-accepting protein IV	-3.25561	0.104704	1.60E-65
ymdA	uncharacterized protein	-3.23283	0.10637	3.96E-44
flgB	flagellar component of cell-proximal portion of basal-body rod	-3.1959	0.109129	3.05E-116
fliT	putative flagellar synthesis and assembly chaperone	-3.18988	0.109585	1.66E-41
intK	Pseudo	-3.18974	0.109595	2.31E-07
flgD	flagellar hook assembly protein	-3.14845	0.112777	8.15E-75
flgK	flagellar hook-filament junction protein 1	-3.13862	0.113548	5.43E-80
ycgR	flagellar velocity braking protein, c-di-GMP-regulated	-3.10105	0.116544	7.22E-80
motB	protein that enables flagellar motor rotation	-3.06355	0.119613	3.44E-51
flgH	flagellar protein of basal-body outer-membrane L ring	-3.02454	0.122892	9.81E-64
cheW	purine-binding chemotaxis protein	-3.00601	0.12448	3.86E-65
flgE	flagellar hook protein	-2.98918	0.125941	1.36E-75
fliE	flagellar basal-body component	-2.98706	0.126126	8.84E-19
yhjH	cyclic-di-GMP phosphodiesterase, FlhDC-regulated	-2.96893	0.127721	4.67E-78
flhB	flagellin export apparatus, substrate specificity protein	-2.96351	0.128202	7.22E-40
fliL	flagellar biosynthesis protein	-2.94132	0.130189	6.43E-82

tsr	methyl-accepting chemotaxis protein I, serine sensor receptor	-2.93408	0.130844	5.04E-63
yehD	putative fimbrial-like adhesin protein	-2.88295	0.135564	0.013735
fliN	flagellar motor switching and energizing component	-2.87561	0.136256	2.09E-39
flgG	flagellar component of cell-distal portion of basal-body rod	-2.85037	0.138661	4.70E-74
cheA	fused chemotactic sensory histidine kinase in two-component regulatory system with CheB and CheY: sensory histidine kinase/signal sensing protein	-2.81554	0.142049	2.06E-56
flgL	flagellar hook-filament junction protein	-2.81371	0.142229	6.40E-65
cspI	Qin prophage; cold shock protein	-2.81137	0.14246	1.60E-10
flxA	Qin prophage; uncharacterized protein	-2.80743	0.14285	9.45E-43
flgJ	flagellar rod assembly protein and murein hydrolase; flagellum-specific muramidase	-2.80327	0.143262	7.14E-57
fliM	flagellar motor switching and energizing component	-2.76244	0.147375	6.70E-58
flgI	putative flagellar basal body protein	-2.70609	0.153245	8.78E-59
cheZ	chemotaxis regulator, protein phosphatase for CheY	-2.6987	0.154032	2.03E-43
fliF	flagellar basal-body MS-ring and collar protein	-2.68638	0.155353	3.05E-54
flgF	flagellar component of cell-proximal portion of basal-body rod	-2.67541	0.156539	1.80E-51
fliI	flagellum-specific ATP synthase	-2.6682	0.157323	6.87E-56
fliJ	flagellar protein	-2.66123	0.158085	2.86E-36
fimA	major type 1 subunit fimbrin (pilin)	-2.64678	0.159676	3.83E-25
ves	cold- and stress-inducible protein	-2.64645	0.159713	3.33E-30
fliG	flagellar motor switching and energizing component	-2.59208	0.165846	8.55E-51
cheB	fused chemotaxis regulator: protein-glutamate methylesterase in two-component regulatory system with CheA	-2.53632	0.172382	4.16E-26
fliQ	flagellar biosynthesis protein	-2.51428	0.175036	1.76E-24
cheY	chemotaxis regulator transmitting signal to flagellar motor component	-2.50114	0.176637	2.88E-40
flhE	proton seal during flagellar secretion	-2.49735	0.177102	3.92E-15
fliO	flagellar biosynthesis protein	-2.45707	0.182116	1.61E-30
pawZ	tRNA	-2.44424	0.183743	0.044885
fliK	flagellar hook-length control protein	-2.44215	0.184009	1.70E-47
yqcG	membrane stress resistance protein	-2.4402	0.184258	0.047623
leuA	2-isopropylmalate synthase	-2.41513	0.187488	1.05E-73
flgN	export chaperone for FlgK and FlgL	-2.39051	0.190715	1.24E-47
flgA	assembly protein for flagellar basal-body periplasmic P ring	-2.38978	0.190811	3.80E-39
fliH	negative regulator of FliI ATPase activity	-2.37769	0.192417	9.01E-41
leuB	3-isopropylmalate dehydrogenase, NAD(+)-dependent	-2.37457	0.192834	2.72E-65
asr	acid shock-inducible periplasmic protein	-2.36831	0.193672	4.45E-25
yjiY	putative transporter	-2.36282	0.194411	3.13E-33
crfC	clamp-binding sister replication fork colocalization protein, dynamin-related	-2.35601	0.195331	4.13E-65
leuD	3-isopropylmalate dehydratase small subunit	-2.34682	0.196579	1.27E-53

flhA	putative flagellar export pore protein	-2.34241	0.197181	1.50E-56
fliP	flagellar biosynthesis protein	-2.3313	0.198705	1.91E-28
leuC	3-isopropylmalate dehydratase large subunit	-2.30845	0.201877	3.53E-68
flgM	anti-sigma factor for FliA (sigma 28)	-2.30738	0.202027	3.34E-46
creD	inner membrane protein	-2.26939	0.207418	2.34E-57
ariR	RcsB connector protein for regulation of biofilm and acid-resistance	-2.26031	0.208727	2.56E-26
ymgC	Blue light, low temperature and stress induced protein	-2.15897	0.223916	6.02E-19
yecR	lipoprotein, function unknown	-2.11405	0.230998	2.72E-26
ycgZ	RcsB connector protein for regulation of biofilm and acid-resistance	-2.07443	0.237429	1.12E-29
yjcZ	YjcZ family protein; yhjH motility defect suppressor	-2.01122	0.248063	6.56E-25
ymgA	RcsB connector protein for regulation of biofilm	-1.94681	0.259389	2.13E-19
aer	fused signal transducer for aerotaxis sensory component/methyl accepting chemotaxis component	-1.9328	0.26192	1.22E-26
fhuE	ferric-rhodotorulic acid outer membrane transporter	-1.90167	0.267633	1.93E-50
shiA	shikimate transporter	-1.89625	0.268641	4.52E-50
cbrA	colicin M resistance protein; FAD-binding protein. putative oxidoreductase	-1.87813	0.272036	3.00E-40
fimB	tyrosine recombinase/inversion of on/off regulator of fimA	-1.83412	0.280463	1.04E-31
leuL	leu operon leader peptide	-1.78529	0.290118	2.74E-09
cbrB	PRK09823 family inner membrane protein. creBC regulon	-1.78367	0.290444	2.73E-32
ydfR	Qin prophage; uncharacterized protein	-1.75447	0.296382	3.95E-05
dmlA	D-malate oxidase. NAD-dependent; putative tartrate dehydrogenase	-1.65867	0.316731	3.09E-26
intG	Pseudo	-1.63084	0.3229	0.000177
yobF	hypothetical protein	-1.62687	0.32379	1.14E-05
ydgU	stationary phase-induced protein	-1.62531	0.32414	0.012689
ydiF	putative acetyl-CoA:acetoacetyl-CoA transferase: alpha subunit/beta subunit	-1.5775	0.335062	2.69E-06
cbrC	UPF0167 family protein	-1.57106	0.336561	3.72E-37
thrC	L-threonine synthase	-1.53188	0.345826	4.29E-31
cspB	Qin prophage; cold shock protein	-1.52687	0.347029	9.23E-05
yiaO	2,3-diketo-L-gulonate-binding periplasmic protein	-1.51828	0.349102	0.016106
thrA	Bifunctional aspartokinase/homoserine dehydrogenase 1	-1.51666	0.349494	1.54E-37
mdtH	multidrug efflux system, subunit A	-1.51315	0.350345	2.55E-12
fabB	3-oxoacyl-[acyl-carrier-protein] synthase I	1.514627	2.857249	1.95E-28
tauA	taurine ABC transporter periplasmic binding protein	1.539718	2.907377	5.12E-10
csrC	ncRNA	1.543237	2.914477	2.53E-18
cpxP	inhibitor of the cpx response; periplasmic adaptor protein	1.547399	2.922897	1.05E-29
ycjP	putative sugar ABC transporter permease	1.570645	2.970375	0.000999
hslO	heat shock protein Hsp33	1.572742	2.974696	2.42E-25
dnaK	chaperone Hsp70, with co-chaperone DnaJ	1.609799	3.052093	8.41E-40
phnI	ribophosphonate triphosphate synthase complex putative catalytic	1.610068	3.052662	0.00265

	subunit			
gadE	gad regulon transcriptional activator	1.610419	3.053405	1.12E-16
ydjO	uncharacterized protein	1.670116	3.182402	5.20E-16
slp	outer membrane lipoprotein	1.725241	3.306354	5.65E-20
dnaJ	chaperone Hsp40, DnaK co-chaperone	1.740763	3.342119	3.33E-42
ycjM	alpha amylase catalytic domain family protein	1.744791	3.351463	1.89E-12
tauC	taurine ABC transporter permease	1.749163	3.361635	2.68E-11
phnD	phosphonate ABC transporter periplasmic binding protein	1.768045	3.405921	1.91E-09
yifB	magnesium chelatase family protein and putative transcriptional regulator	1.786701	3.45025	7.38E-32
ompC	outer membrane porin protein C	1.798421	3.478393	3.07E-24
srlA	glucitol/sorbitol-specific enzyme IIC component of PTS	1.838098	3.575384	4.49E-06
arrS	ncRNA	1.872607	3.661937	1.13E-07
groL	Cpn60 chaperonin GroEL, large subunit of GroESL	1.877091	3.673336	9.79E-52
yneL	Pseudo	1.934161	3.821558	0.045852
gspI	general secretory pathway component, cryptic	1.937428	3.830222	0.005779
rrsA	16S ribosomal RNA of rrnA operon	1.985039	3.958734	1.85E-05
trpD	fused glutamine amidotransferase (component II) of anthranilate synthase/anthranilate phosphoribosyl transferase	1.995814	3.988411	1.34E-12
groS	Cpn10 chaperonin GroES, small subunit of GroESL	2.007288	4.020258	5.88E-57
yjaB	GNAT-family putative N-acetyltransferase; acetyl coenzyme A-binding protein	2.008696	4.024183	1.09E-10
gltV	tRNA	2.056278	4.159119	1.92E-09
gadA	glutamate decarboxylase A, PLP-dependent	2.111414	4.321146	1.21E-29
caiE	stimulator of CaiD and CaiB enzyme activities	2.131001	4.380213	0.000303
hdeD	acid-resistance membrane protein	2.144767	4.422208	9.96E-40
mdtE	anaerobic multidrug efflux transporter, ArcA-regulated	2.149699	4.437352	3.03E-27
phnC	phosphonate ABC transporter ATPase	2.283205	4.867581	6.97E-13
srlB	glucitol/sorbitol-specific enzyme IIA component of PTS	2.318971	4.989762	1.15E-06
alaU	tRNA	2.33104	5.031679	0.002696
dctR	Putative LuxR family repressor for dicarboxylate transport	2.349665	5.097059	8.30E-22
gadC	glutamate:gamma-aminobutyric acid antiporter	2.41741	5.342111	4.44E-36
omrA	ncRNA	2.435541	5.409672	0.016323
ssuD	alkanesulfonate monooxygenase, FMNH(2)-dependent	2.526244	5.760699	1.96E-15
ssuE	NAD(P)H-dependent FMN reductase	2.534243	5.792728	5.79E-15
trpE	component I of anthranilate synthase	2.542093	5.824334	2.73E-15
ssuC	aliphatic sulfonate ABC transporter permease	2.555359	5.878137	1.79E-09
hdeA	stress response protein acid-resistance protein	2.57932	5.976579	1.17E-32
pyrI	aspartate carbamoyltransferase, regulatory subunit	2.791375	6.922893	6.83E-81
ssuA	aliphatic sulfonate ABC transporter periplasmic binding protein	2.802741	6.977649	2.43E-24
gadB	glutamate decarboxylase B, PLP-dependent	2.852421	7.222113	1.23E-36

ileT	tRNA	2.963274	7.798918	0.001226
hdeB	acid-resistance protein	2.976797	7.872364	3.12E-70
pyrB	aspartate carbamoyltransferase, catalytic subunit	2.992486	7.958442	2.23E-115
yhiD	putative Mg(2+) transport ATPase, inner membrane protein	3.546028	11.68048	1.16E-40
mtr	tryptophan transporter of high affinity	3.946395	15.41641	1.94E-20
rrsE	16S ribosomal RNA of rrnE operon	4.745603	26.8268	0.012375
rrfA	5S ribosomal RNA of rrnA operon	7.454931	175.4518	8.48E-18
rrfB	5S ribosomal RNA of rrnB operon	9.125841	558.6655	0

Table 6.6 Gene ontology (GO) analysis of the differentially expressed genes that are downregulated in cells expressing the mirror stem loop stem sequence. Overrepresented processes are indicated with "+", underrepresented process with "-".

Term	Background frequency	Sample frequency	Expected	+/-	P-value
locomotion (GO:0040011)	71	36	1.652e+00	+	3.026e-35
localization of cell (GO:0051674)	43	29	1.000e+00	+	4.396e-31
cell motility (GO:0048870)	43	29	1.000e+00	+	4.396e-31
archaeal or bacterial-type flagellum-dependent cell motility (GO:0097588)	37	27	8.607e-01	+	1.312e-29
cilium or flagellum-dependent cell motility (GO:0001539)	37	27	8.607e-01	+	1.312e-29
movement of cell or subcellular component (GO:0006928)	52	29	1.210e+00	+	9.433e-29
bacterial-type flagellum-dependent cell motility (GO:0071973)	35	25	8.142e-01	+	5.848e-27
taxis (GO:0042330)	26	18	6.048e-01	+	1.794e-18
chemotaxis (GO:0006935)	26	18	6.048e-01	+	1.794e-18
bacterial-type flagellum organization (GO:0044781)	16	11	3.722e-01	+	1.659e-10
response to external stimulus (GO:0009605)	115	20	2.675e+00	+	1.919e-09
single-organism organelle organization (GO:1902589)	27	11	6.281e-01	+	4.259e-08
bacterial-type flagellum assembly (GO:0044780)	13	8	3.024e-01	+	8.600e-07
cell projection organization (GO:0030030)	37	11	8.607e-01	+	1.128e-06
cell projection assembly (GO:0030031)	18	8	4.187e-01	+	1.057e-05
metabolic process (GO:0008152)	2371	28	5.515e+01	-	1.685e-05
localization (GO:0051179)	758	41	1.763e+01	+	2.676e-05
organelle organization (GO:0006996)	57	11	1.326e+00	+	8.955e-05
response to chemical (GO:0042221)	298	23	6.932e+00	+	2.252e-04
organelle assembly (GO:0070925)	28	8	6.513e-01	+	2.998e-04
organic substance metabolic process (GO:0071704)	2034	24	4.731e+01	-	8.274e-04
leucine biosynthetic process (GO:0009098)	8	5	1.861e-01	+	1.237e-03
leucine metabolic process (GO:0006551)	8	5	1.861e-01	+	1.237e-03

cellular metabolic process (GO:0044237)	2016	24	4.690e+01	-	1.249e-03
single-organism cellular process (GO:0044763)	1736	61	4.038e+01	+	1.555e-02
primary metabolic process (GO:0044238)	1783	22	4.148e+01	-	2.305e-02
biological adhesion (GO:0022610)	51	8	1.186e+00	+	2.350e-02
establishment of localization in cell (GO:0051649)	40	7	9.305e-01	+	3.860e-02
single-organism metabolic process (GO:0044710)	1449	16	3.371e+01	-	4.391e-02

Table 6.7 GO analysis of differentially expressed genes that are upregulated in cells expressing the mirror stem loop stem sequence. Overrepresented processes are indicated with "+", underrepresented process with "-".

Term	Background frequency	Sample frequency	Expected	+/-	P-value
response to abiotic stimulus (GO:0009628)	142	9	1.445e+00	+	9.189e-03
pH elevation (GO:0045852)	5	3	5.088e-02	+	1.687e-02
intracellular pH elevation (GO:0051454)	5	3	5.088e-02	+	1.687e-02
response to heat (GO:0009408)	57	6	5.801e-01	+	2.034e-02
regulation of intracellular pH (GO:0051453)	6	3	6.106e-02	+	2.894e-02
protein refolding (GO:0042026)	7	3	7.124e-02	+	4.563e-02

In **Table 6.5**, a list is given of the genes that are differentially expressed in strains that express the mirror stem loop stem sequence compared to cells with an empty vector. Many of the genes are part of an operon, supporting the reliability of the data. An effect on the expression of essential genes was expected, which would explain the reduced growth. Remarkably, a lot of genes that also have a function in biofilm development came up as being differentially expressed, although the samples were taken in planktonic phase.

The genes that have the largest reduction in expression are the curli specific genes (*csg*), comprising two operons involved in curli production, the *csgBAC* operon and the *csgDEFG* operon. Curli are mainly required for biofilm formation in many enterobacteria, promoting surface adherence and cell aggregation as they are the major proteinous components of the extracellular matrix (Barnhart & Chapman, 2006). CsgD also indirectly regulates the production of cellulose, another matrix component as the protein transcriptionally activates *adrA* and *yoaD*, encoding proteins involved in c-di-GMP metabolism. c-di-GMP, on its turn, is necessary for the production of cellulose (Barnhart & Chapman, 2006). Because of its involvement in curli and cellulose production, CsgD is considered to be the central regulator of biofilm development. A downregulation of *csgD* expression might therefore explain why cells expressing the mirror stem loop stem have a reduced biofilm forming capacity. Additional genes involved in matrix production that are downregulated in the presence of

the mirror sequence are *ymgA* and *ariR*. The encoded proteins activate the production of the matrix substance colonic acid, but are also involved in the activation of acid resistance genes (Tschowri *et al.*, 2009). Other genes that show a reduced expression level in the presence of the mirror sequence are mainly involved in motility and chemotaxis (see **Table 6.6**). It appears that motility is also important for biofilm development, at least at the early stages of initiating biofilm formation (Guttenplan & Kearns, 2013). Therefore, a reduced motility might be another reason why initiation of biofilm formation is affected in strains expressing the mirror stem loop stem sequence.

For the genes that are upregulated, there is an enrichment for genes involved in response to pH stress (see **Table 6.7**). Activating stress genes might slow down the growth of the strain. Additionally, some genes encoding ribosomal RNA and tRNA, such as *rrfB*, *rrfA*, *rrsE*, *ileT*, *alaU* and *gltV* are highly upregulated.

Related to CsrA, two genes are interesting in the list of differentially expressed genes. These are *yjiY* and *csrC*. *yjiY* is another identified CsrA target (Behr *et al.*, 2014) the expression of which is downregulated in the presence of the mirror stem loop stem sequence, while *csrC* expression, involved the regulation of CsrA activity (Weilbacher *et al.*, 2003) is upregulated under the same conditions. Other known CsrA targets were not differentially expressed, confirming previous results and suggesting that the mirror stem loop stem sequence indeed has another working mechanism compared to the sense stem loop stem sequence.

6.3.4 Validation of RNA-seq data

The RNA-seq data reveal that curli genes and flagella genes are downregulated in the presence of the mirror stem loop stem sequence. This observation was validated by measuring the expression levels of *csgD*, *csgB*, *fliZ*, *fliA* and *motB* with qRT-PCR. Additionally, the expression levels of *rrfA*, *gadB* and *hdeB* was determined. Their expression was shown to be upregulated in the RNA-seq data. The results are shown in **Figure 6.4**.

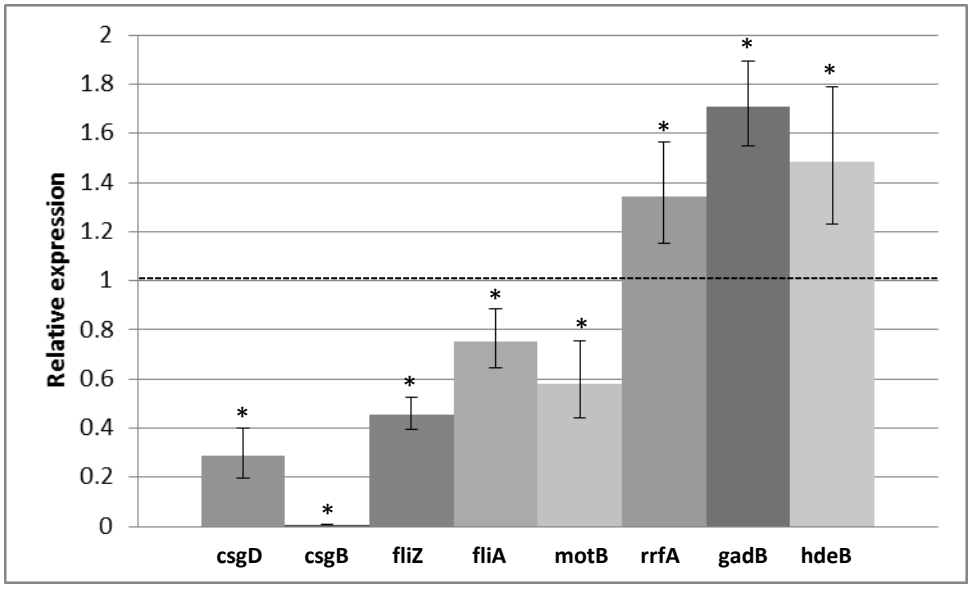


Figure 6.4 Relative expression levels of *csgD*, *csgB*, *fliZ*, *fliA*, *motB*, *rrfA*, *gadB* and *hdeB* in cells expressing the mirror stem loop stem sequence (pCMPG10901) compared to cells with a control plasmid (pCMPG10908) for cells grown in 1/20 TSB at 25°C. Error bars represent 95% confidence intervals. A star indicates that the C_T values are significantly different for both strains ($p < 0.05$). The dotted line indicates a relative expression level of 1. Relative expression levels > 1 indicate increased expression, relative expression levels <1 indicate reduced expression compared to the control strain.

The results in **Figure 6.4** confirm that *csgD, csgB, fliA, fliZ* and *motB* expression is downregulated when the mirror stem loop stem sequence is expressed. *rrfA*, *gadB* and *hdeB* expression is upregulated. These results confirm the results of the RNA-seq experiment. Although the relative differences in expression are smaller when measured with qRT-PCR, the trend is similar.

Additionally, to confirm that motility genes are downregulated in the presence of the mirror sequence, a swarming assay was done. Swarming motility is defined as a rapid multicellular bacterial surface movement powered by rotating flagella (Henrichsen, 1972; Kearns, 2011). Therefore, if the motility genes are downregulated, swarming is expected to be reduced. The results are shown in **Figure 6.5**.

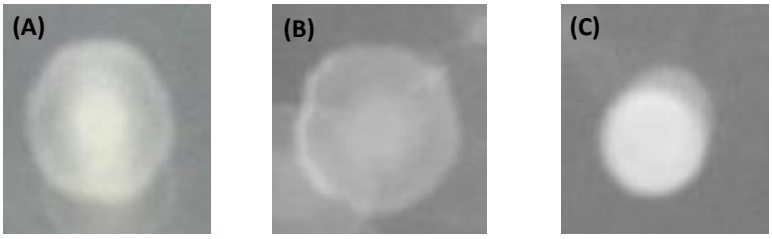


Figure 6.5 Swarming assay of **(A)** DH5α WT, **(B)** DH5α expressing a random sequence (pCMPG10907) or **(C)** the mirror stem loop stem sequence (pCMPG10901)**.** Cells were grown on swarming agar at 30 °C.

The results in **Figure 6.5** show that swarming is reduced in the presence of the mirror stem loop stem sequence (pCMPG10901) if compared to the wild type or strains expressing a random sequence (pCMPG10907).

6.3.5 Network analysis

The RNA-seq analysis identified genes of which the expression is different in strains expressing the mirror stem loop stem sequence and strains with an empty vector. However, to get insight into the molecular interactions of the differentially expressed genes and into the underlying biology that drives these gene expression differences, the gene expression data were further analyzed by constructing an interaction network using the PheNetic website (De Maeyer *et al.*, 2013). PheNetic uses the interaction network of *E. coli*, which is based on publically available interactomics data, and integrates different layers of information, such as molecular interactions and signaling layers of more than 16 000 physical or metabolic interactions, covering protein-protein interactions, transcriptional and metabolic interactions. The program searches within the interaction network for active paths that explain the effect of one or more nodes (causes) on one or multiple modes downstream (effect), revealing the response mechanism connecting the nodes. The inferred network is shown in **Figure 6.6**.

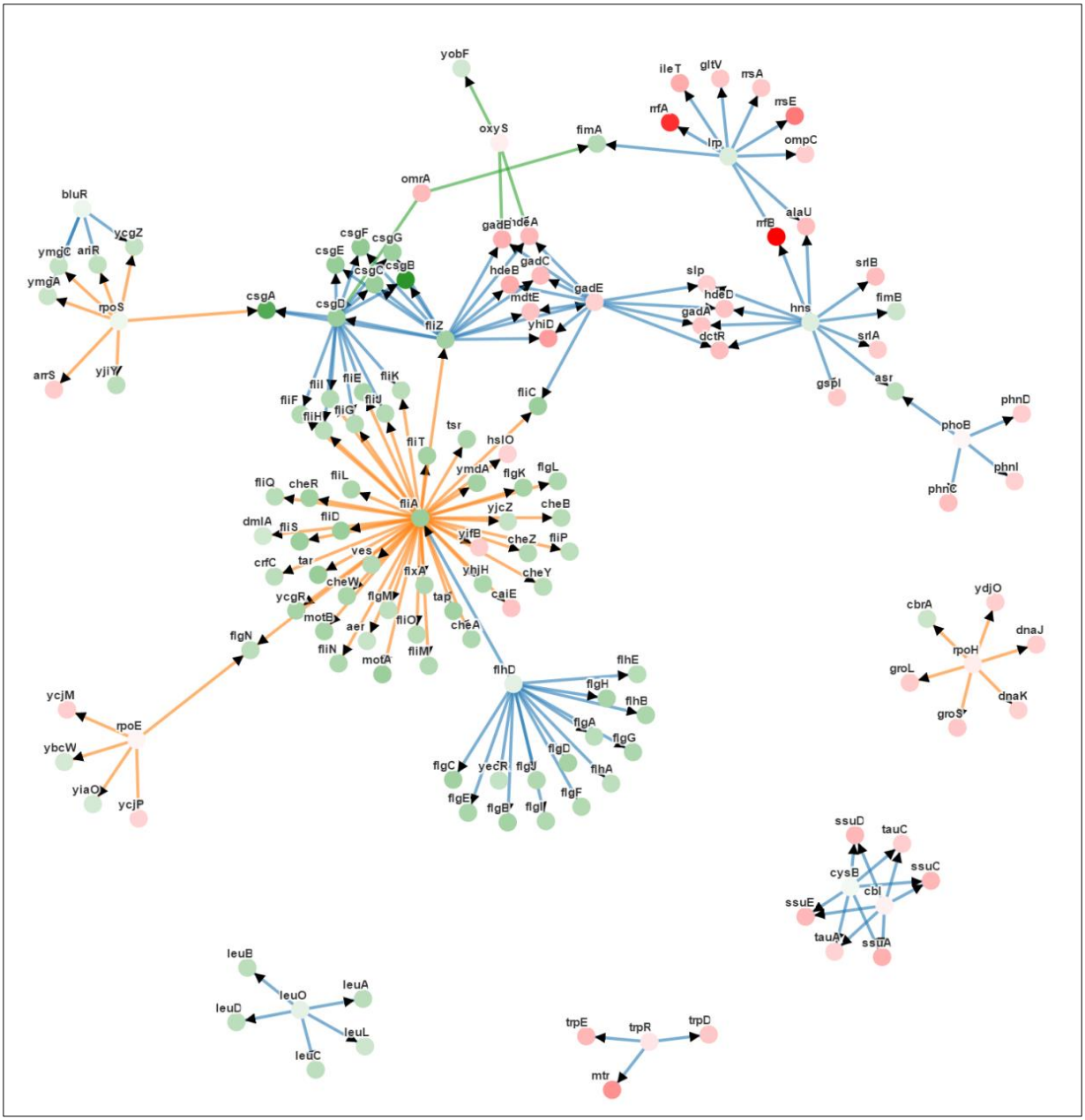


Figure 6.6 Network analysis of the differentially expressed genes between strains expressing the mirror stem loop stem sequence and strains with an empty plasmid using the PheNetic software. Green dots indicate that the expression of the gene is downregulated, red dots indicate that its expression is upregulated, the more intense, the higher the log fold change. Orange arrows represent protein/DNA interactions, blue arrows represent protein/protein interactions and green arrows represent post-transcriptional interactions.

According to the PheNetic network, genes involved in the assembly of the flagellar basal body and in the flagellar export (*fli*, *flg* and *flh* genes, including the flagellar σ subunit (*fliA*), and its anti-σ factor, *flgM*), flagellar rotation (*mot* genes), chemotactic membrane receptors (*tar*, *tsr*, *tap*, *trg* genes) and

chemotactic signal transduction (*che* genes) are all regulated by FlhD. Biologically, it is $FlhD_2C_2$, a heterotrimeric DNA binding transcriptional regulator, that is the active molecule regulating target gene expression. $FlhD_2C_2$ is necessary for the activation of the different genes involved in flagellar synthesis, assembly and activity. It also activates *fliA* expression, a sigma factor that also positively regulates transcription of flagellar genes and additionally affects *flhDC* transcription positively. Therefore, gene expression differences in all of these genes can be attributed to differences in $FlhD_2C_2$ expression or activity. Similarly, expression differences in the curli specific genes (*csgBAC* and *csgDEFG*) can be attributed to CsgD expression differences.

Furthermore, genes of the major glutamate-dependent acid resistance system, involved in maintenance of pH homeostasis, including two glutamate decarboxylase paralogues *gadA* and *gadB*, the glutamate/gamma-aminobutyrate antiporter *gadC*, the acid stress chaperones *hdeA* and *hdeB*, the membrane protein *hdeD*, the transcriptional regulator *yhiF* (*dctR*) and the gene encoding the outer membrane protein *slp*, are all regulated by the transcriptional regulator GadE (Krin *et al.*, 2010a). Additionally, the expression of some of these genes, such as *slp* and *dctR*, and *gadE* itself is directly repressed by H-NS (Krin *et al.*, 2010b). H-NS is the histon-like nucleoid structuring protein that affects the expression of at least 250 genes. Remarkably, H-NS also promotes motility via FlhDC by repressing RcsB (Krin *et al.*, 2010a). Therefore, a reduced activity of H-NS would explain the upregulation of GadE and the downregulation of FlhDC, together with their respective targets, which is a large part of the differentially expressed genes.

Other genes, such as *ileT*, *rrsA*, *gltV* and *rrsE*, are all regulated by the transcriptional regulator Lrp. This is a leucine responsive regulatory protein and is a master regulator of gene expression. The protein can both act as a repressor or as an activator, wherein leucine can be necessary for or can overcome the regulation by Lrp (Chen *et al.*, 2005). Finally, the expression of *tauABCD* and *ssuEADCB* are commonly regulated by CysB and Cbl. These genes encode an ABC-transporter for the uptake of aliphatic sulphonates and a desulfonation enzyme, enabling the use of sulphonates as a sulfur source in the absence of sulphate and cysteine. Synthesis of Cbl itself is under control of CysB, therefore CysB is thought to be the master regulator in this process (van der Ploeg *et al.*, 2001). CysB regulates the expression of the *cys* genes in the assimilatory sulphate reduction pathway, but these genes were not identified as being differentially expressed. Regulators that control a smaller number of differentially expressed genes are *rpoS*, *rpoE*, *leuO*, *bluR* and *phoB*.

A remarkable observation in the gene expression data is that both the expression of flagellar genes and expression of *csgD* is downregulated. The expression of *fliZ* and *csgD* is generally assumed to be

oppositely regulated, illustrated by the repressive activity of CsgD on flagellar operons, as also shown on the interaction network (Ogasawara *et al.*, 2011). Nevertheless, the downregulation of both processes might be explained by the upregulation of the sRNA *omrA*. Overexpression of *omrA* reduces curli formation through inhibiting *csgD* expression. Additionally it binds to the *flhDC* mRNA near the Shine-Dalgarno region, thereby reducing its expression (Mika & Hengge, 2014). However, OmrA is also shown to negatively regulate *ompT*, *cirA*, *fecA*, *fepA* and *ompR* next to *flhDC* and *csgD*, which cannot be observed in the gene expression data. Additionally, it is unclear why OmrB, which is a sRNA with a redundant function compared to OmrA (Guillier & Gottesman, 2008), is not upregulated. Another regulator that might explain the downregulation of both *csgD* and flagellar gene expression is CpxR as CpxR represses *csgD* expression by binding near the promoter region (Jubelin *et al.*, 2005) and indirectly represses flagellar biosynthesis (Dudin *et al.*, 2014). However, in the presence of the mirror sequence, *cpxR* is not differentially expressed. Moreover, another component in this regulatory network, *cpxP*, is upregulated, which generally leads to a downregulation of the cpx response (Fleischer *et al.*, 2007). Therefore, the involvement of this regulator in the activity of the mirror sequence is unlikely.

In conclusion, some of the differentially expressed genes are controlled by the same regulator, suggesting that differences in this regulator are responsible for the expression differences observed in downstream genes. Therefore, these genes are more likely to be the target of the mirror stem loop stem sequence.

6.3.6 Analysis of the binding requirements of the identified regulators

Based on the fact that changes in the activity or expression of certain regulators would explain why multiple genes are differentially expressed in the presence of the mirror stem loop stem sequence, it might be that the sequence is active by competing with other targets for binding to the regulatory protein, similar to the mode of action of the sense stem loop stem sequence. Therefore, the binding preferences of the central regulators, identified in the PheNetic network, are compared to the mirror sequence (see **Table 6.8**).

Table 6.8 Binding requirements of the central regulators of the genes that are differentially expressed between strains expressing the mirror stem loop stem sequence and strains with an empty vector and their respective consensus binding site. Also the mirror stem loop stem sequence is given.

Regulator	Consensus binding site	Reference
CsgD	AAAAGNGNNAAAWW	(Dudin *et al.*, 2014)
CpxR box	GTAAA(N_5)GTAAA	(De Wulf *et al.*, 2002)
FlhDC	tNAAcGCc(N)$_2$AAATAgcg	(Zhao *et al.*, 2007)
Gad box	TTAGGATTTTGTTATTTAAA	(Castanie-Cornet & Foster, 2001)
H-NS	tCG(t/a)T(a/t)AATT	(Sette *et al.*, 2009)
Lrp	AGAATTTTATTCT	(Cui *et al.*, 1995)
OmrA	CCCAGAGGUAUUGAUAGGUGA	(Holmqvist *et al.*, 2010)
Mirror stem loop stem	UGUAGGAACAUGCTGCAG	This work

Based on the binding preferences, shown in **Table 6.8**, it is difficult to predict which regulator might be targeted by the mirror stem loop stem sequence. To be able to make a more solid prediction, the mirror stem loop stem sequence is characterized to identify the nucleotides that are important for the negative effect on the biofilm forming capacity of the cells expressing the mirror stem loop stem sequence.

6.3.7 Characterization of mirror stem loop stem sequence

To identify the important nucleotides in the sequence, some mutations were constructed in the mirror stem loop stem sequence. Firstly, the effect of the restriction site, present in the plasmid that encodes the mirror sequence, was evaluated. Hereto, the mirror stem loop stem sequence was expressed from a plasmid containing an *EcoRI* sequence (pCMPG10912) and the effect on biofilm formation was compared to cells expressing the mirror stem loop stem sequence from a plasmid containing a *PstI* restriction site (pCMPG10901), for which the results were already shown in **section 3.3.3**. The results are shown in **Figure 6.7**.

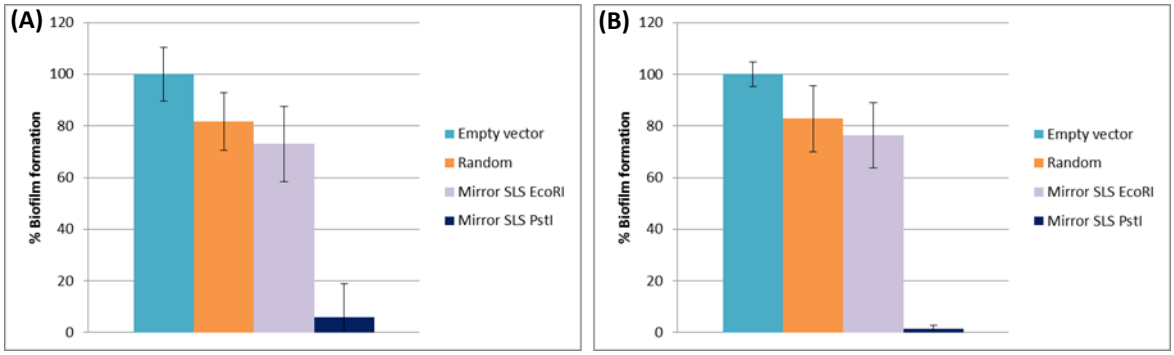


Figure 6.7 Biofilms formed by *E. coli* DH5α strains with plasmids expressing the mirror sequence with different restriction sites (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), short mirror stem loop stem with *EcoRI* restriction site (pCMPG10912), short mirror stem loop stem with *PstI* restriction site (pCMPG10901). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

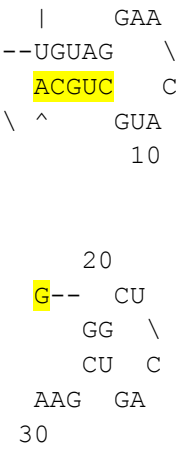
From **Figure 6.7** it is clear that, contrary to the results for the sense construct (see **Figure 4.1**), the mirror sequence only has an effect on the amount of biofilm that is formed when it is expressed from a plasmid that was constructed with a *PstI* restriction site (pCMPG10901) and not when it was constructed with an *EcoRI* restriction site (pCMPG10912). Possibly the nucleotides of the *PstI* restriction site are important for the direct interaction with the target or they influence the secondary structure of the sequence, which might influence target binding as well.

To examine whether the nucleotides of the restriction site influence the secondary structure, the structure of the mirror stem loop stem sequence with an *EcoRI* and *PstI* restriction site was predicted with the online structure prediction tool mFOLD (Zuker, 2003). The results are shown in **Figure 6.8**.

Mirror stem loop stem *PstI* (pCMPG10901)

Structure 1 Folding bases 1 to 30

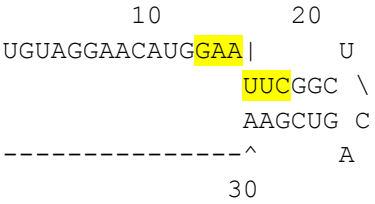
Initial ΔG = -4.80



Mirror stem loop stem *EcoRI* (expressed from pCMPG10912)

Structure 1 Folding bases 1 to 30

Initial ΔG = -3.80



Structure 2 Folding bases 1 to 30

Initial ΔG = -3.10

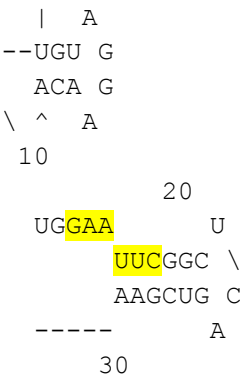


Figure 6.8 Secondary structure prediction using the mFOLD algorithm of the mirror stem loop stem sequence with and *PstI* restriction site (pCMPG10901) and with a *EcoRI* restriction site (pCMPG10912). The restriction site is indicated in yellow.

Figure 6.8 shows that the mirror sequence containing a *PstI* restriction site is predicted to form a structure in which the nucleotides of the restriction site base pair to the nucleotides of the mirror sequence, resulting in a single stranded loop of seven nucleotides. The mirror sequence containing an *EcoRI* restriction site can form two different structures with comparable free energy, in which the nucleotides of the restriction site base pair with nucleotides of the terminator region of the plasmid. Therefore, the predicted secondary structure is different dependent on the restriction site present in the construct. Consequently, it is possible that the secondary structure plays a role in target interaction and anti-biofilm activity.

To confirm the importance of the secondary structure, nucleotides at the base of the predicted stem are mutated, because often they are important for the stability of the stem loop. Therefore, the GGA in the sequence was mutated to TTA (pCMPG10924) and CCA (pCMPG10925). Mutating these nucleotides indeed inhibits the anti-biofilm effect, suggesting that it is indeed the secondary structure that is important (see **Figure 6.9**).

Additionally, all nucleotides, except the AGGA and the nucleotides of the restriction site, were mutated to C or G (pCMPG10926). These changes inhibited the anti-biofilm effect (see **Figure 6.9**), showing that although the AGGA and the sequence of the restriction site are essential to reduce biofilm formation, the presence of these nucleotides is not sufficient for the anti-biofilm effect. This suggests that the interaction with other nucleotides in the sequence, making up the secondary structure, is indeed important.

Lastly, nucleotides in the loop of the predicted secondary structure were changed (pCMPG10927 and pCMPG10928). These mutations enhanced the anti-biofilm effect, suggesting that the sequence in the loop is not essential for the reduced effect on biofilm formation (see **Figure 6.9**). An overview of all mutated sequences tested, is given in **Table 6.9**.

Table 6.9 Mutated mirror stem loop stem sequences and their effect on biofilm formation. The nucleotides that are different compared to sequence of the original mirror stem loop stem sequence (pCMPG10901) are indicated in bold.

Description	Sequence	Effect on biofilm formation
Mirror SLS *PstI* - pCMPG10901	UGUAGGAACAUGCUGCAG – terminator	Negative effect
Mirror SLS *EcoRI* - pCMPG10912	UGUAGGAACAU**GGAAUUC** – terminator	Restoration wt
Mirror SLS mut1 *PstI* - pCMPG10924	UGUA**UU**AACAUGCUGCAG – terminator	Restoration wt
Mirror SLS mut2 *PstI* - pCMPG10925	UGUA**CC**AACAUGCUGCAG – terminator	Restoration wt
Mirror SLS mut3 *PstI* - pCMPG10926	**GGGG**AGGA**CCCC**CUGCAG – terminator	Restoration wt
Mirror SLS mut4 *PstI* - pCMPG10927	AGUAG**CU**A**AGA**GCUGCAG – terminator	Negative effect
Mirror SLS mut5 *PstI* - pCMPG10928	AGUAGG**C**A**UUCA**CUGCAG – terminator	Negative effect

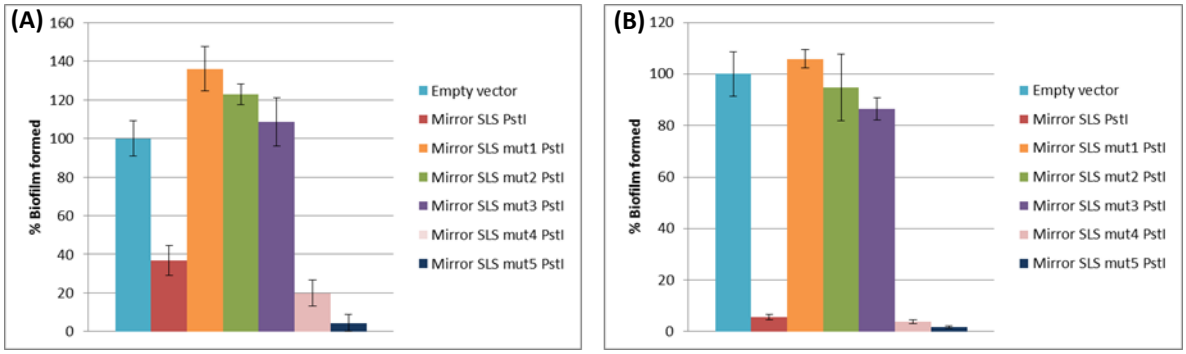


Figure 6.9 Biofilms formed by *E. coli* DH5α strains plasmids expressing the mirror sequence in which different nucleotides are changed (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing the mirror stem loop stem sequence (pCMPG10901) and different mutated mirror stem loop stem sequences (pCMPG10924 – pCMPG10928). In Mirror SLS mut1 (pCMPG10924) the GGA at the base of the predicted stem is mutated to TTA, in Mirror SLS mut2 (pCMPG10925) the GGA is mutated to CCA. In Mirror SLS mut3 (pCMPG10926) all nucleotides except for GGA and the restriction site are mutated to G or C, in Mirror SLS mut4 (pCMPG10927) and Mirror SLS mut5 (pCMPG140928), the sequences in the loop of the structure predicted by mFOLD are mutated. All sequences are expressed from a plasmid constructed with a *PstI* restriction site. Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

By combining our knowledge about the properties of the mirror stem loop stem sequence, which are important for the anti-biofilm effect (see **Table 6.9**), and the binding requirements of the regulators identified with PheNetic (see **Table 6.8**), Lrp can be suggested as a good candidate target of the

mirror stem loop stem sequence. Indeed, the recognition sequence of this regulator comprises a central AT-rich sequence that is flanked by CTG/CAG triplets, which corresponds to the nucleotides identified to be important for the mirror sequence. This suggests that the mirror stem loop stem sequence can indeed compete with the normal targets of Lrp. Moreover, Lrp is a general regulator, influencing the expression of a lot of genes. This might explain the reduced growth of the strain (Ambartsoumian *et al.*, 1994).

However, possibly this is not the only effect of the mirror sequence. Although we suspect the sequence to affect the activity of a protein because the secondary structure of the sequence seems to be important, it is possible that the sequence binds to RNA molecules. These can be mRNAs, preventing their expression or sRNAs, preventing their regulatory action. Remarkably, the mirror sequence resembles the mRNA of *flhD* near the translation start site (see **Figure 6.10**). Therefore, the downregulation of the motility genes might also be explained by the mirror sequence that titrates away an activating sRNA. However, the only FlhDC-activating sRNA that was thus far identified is McaS and this sRNA binds more upstream in the 5' leader of the FlhD transcript. Because the sequence is AG-rich, resembling a Shine-Dalgarno region, and because of the presence of AUG and CUG, similar to a startcodon, the sequence resembles the translation start region of multiple other genes and might therefore bind to other sRNAs that normally bind to the translation start region of genes.

```
                              +1
5'UTR            UUAUUCUGGGUGGGAAUA**AUG**CAUACCUCCGAG 33
mirror           ------UGUAGGAACAUGCUGCAG--------- 18
                       **   *   **  ****
```

Figure 6.10 Alignment of the mirror stem loop stem sequence with a *PstI* restriction site (pCMPG10901) and the *flhD* mRNA near the translation start site.

6.3.8 Expanding the analysis to *E. coli* TG1 and *S.* Typhimurium

Similar to the experiments in **section 4.3.3**, the effect of the mirror stem loop stem sequence was also tested in *E. coli* TG1 and in *S.* Typhimurium. The results are shown in **Figure 6.11** and **6.12**, respectively.

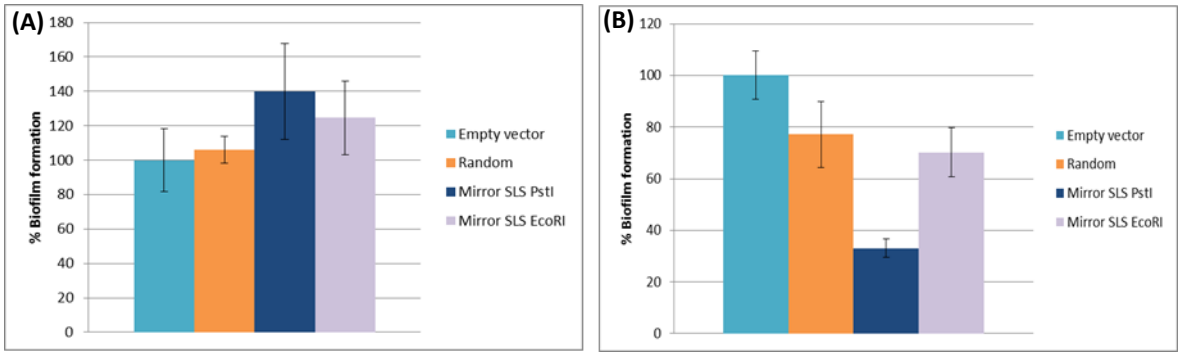


Figure 6.11 Biofilms formed by *E. coli* TG1 strains with plasmids expressing the mirror sequence with different restriction sites (A) after 24 hours and (B) after 48 hours of growth. The results are shown for strains with the empty vector (pCMPG10908) and for strains expressing a random sequence (pCMPG10907), short mirror stem loop stem with *PstI* restriction site (pCMPG10901) and a short mirror stem loop stem with *EcoRI* restriction site (pCMPG10912). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

In **Figure 6.11**, it can be seen that only the mirror stem loop stem sequence expressed from a plasmid constructed with a *PstI* restriction site (pCMPG10901), negatively affects biofilm formation of *E. coli* TG1, but only after 48 hours of growth. The effect on biofilm formation is also smaller in *E. coli* TG1 than in *E. coli* DH5α.

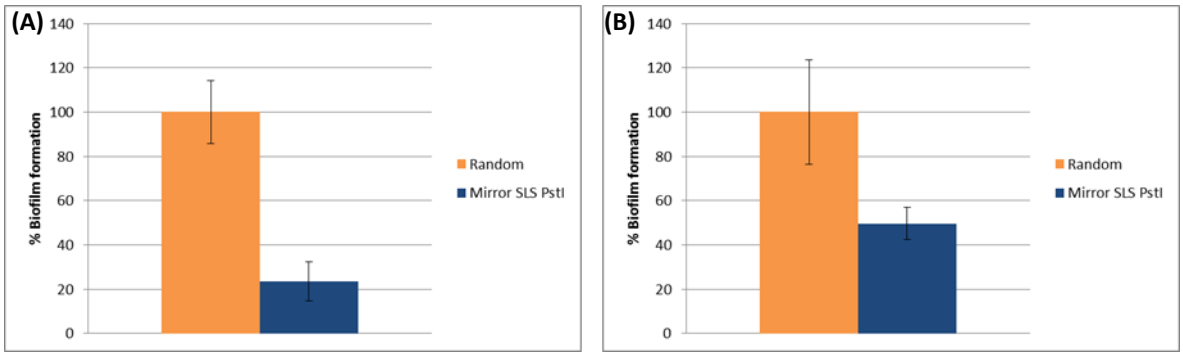


Figure 6.12 Biofilms formed by *Salmonella* Typhimurium SL1344 strains with plasmids expressing the mirror sequence with different restriction sites (A) after 24 hours and (B) after 48 hours of growth. The strains were grown in the Calgary Biofilm Device in 1/20 TSB at 25°C and results are shown for strains expressing a random sequence (pCMPG10907), short mirror stem loop stem with *PstI* restriction site (pCMPG10901). Error bars represent standard deviations. The amount of biofilm formed by strains with the empty plasmid (pCMPG10908) is set at 100 %.

Figure 6.12 shows that biofilm formation is also reduced when the mirror stem loop stem sequence is expressed in *S.* Typhimurium. However, in this bacterium, growth is significantly affected in the presence of the sequence, even in the overnight preculture in LB (see **Figure 6.13**). This might be important to explain the reduced capacity to form biofilms. Remarkably, after 48 hours of incubation, the negative effect on biofilm formation is reduced compared to the results after 24 hours of growth. This is contrary to the results of the biofilm assays of all other sequences and strains tested so far, for which the negative effect on biofilm is enlarged in function of time. This indicates that the negative effect on biofilm formation can be predominantly attributed to the growth effect and the sequence itself might even have a positive effect on biofilm formation in *S.* Typhimurium.

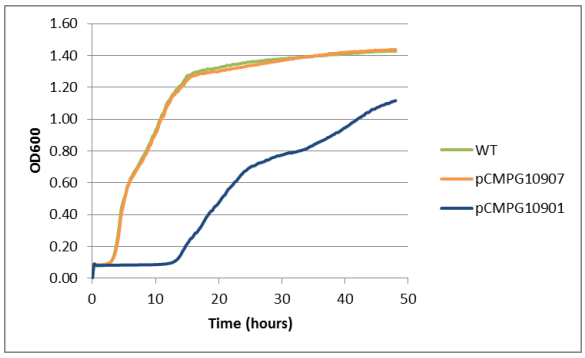


Figure 6.13 Growth of *Salmonella* Typhimurium strains with plasmids expressing the mirror sequence grown in LB at 37°C, continuously monitored with the Bioscreen. The results are shown for the *S.* Typhimurium wild type and for *S.* Typhimurium strains expressing a random sequence (pCMPG10907) and the mirror stem loop stem (pCMPG10901).

6.4. Discussion

In **Chapter 3**, the mirror stem loop stem sequence was introduced as a negative control in the search for sequences that disturb the CsrA-based regulatory network. Unexpectedly, this mirror stem loop stem sequence was shown to affect both biofilm formation and growth when expressed from a plasmid. The CFU count revealed that expressing the sequence causes a delay in both planktonic growth and formation of the biofilm, resulting in a reduced number of biofilm cells after 24 hours of

incubation. Consequently, the negative effect on growth might play an important role in the effect on biofilm formation.

Searching for the mode of action of the mirror stem loop stem sequence, a gene expression analysis showed that CsrA activity is not reduced when the mirror sequence is expressed, in contrast to the effect of the sense stem loop stem sequence. This was already suspected because the sequence has less resemblance to the consensus CsrA binding site than the sense sequence, but has a stronger effect on biofilm development. Therefore, an open approach was used to unravel which processes and pathways are affected by the sequence. An RNA-seq analysis identified genes that were differentially expressed between strains expressing the mirror stem loop stem sequence and strains with an empty vector. Although the gene expression differences are not based on expression data from biofilm cells, samples from the planktonic state can also reveal interesting information. Indeed, because the sequence reduces growth, it clearly has a target during planktonic growth. This target might also affect biofilm development. Moreover, this reduced growth might also be one of the reasons why also biofilm development is affected. Additionally, because gene expression has to be adapted in planktonic phase before cells go into surface-attached mode of growth, some of the genes important for biofilm formation are aready expressed in planktonic growth and consequently, the effect on the expression of these genes can also be detected. Indeed, among the genes that were downregulated, several play a role in biofilm development as well as in planktonic growth. For instance, genes involved in motility (*flh*, *flg* and *mot* genes) are important for cellular movement during planktonic growth, but are also necessary for initiation of biofilm formation. Additionally, the expression of curli specific genes (*csg*), which are important for the production of certain matrix components, is affected. The downregulation of these genes might be linked to the impaired biofilm development. The genes that were upregulated in the presence of the mirror sequence were enriched for genes responsive to low pH. Activation of these stress genes might slow down the growth. However, because these are not data from biofilm cells, it is possible that the main target under biofilm conditions is not expressed under the conditions tested and will consequently not be identified here.

Remarkably, some ribosomal RNA and tRNA encoding genes were identified as being upregulated as well. Normally, the rRNA and tRNA is removed from the RNA samples before the library for sequencing is constructed. Therefore, it is possible that the ribosomal RNA was not removed sufficiently in the RNA sample of strains expressing the mirror stem loop stem sequence, or these reads have been wrongly mapped onto the genome. The effect of the some of the differentially expressed genes in the presence of the mirror stem loop stem sequence was validated with qRT-PCR.

Additionally, the reduced expression of the flagellar genes was confirmed with a swarming assay. However, the reduced growth might affect swarming behavior as well.

Based on the differentially expressed genes, some hypotheses can be formulated regarding the targets and the mode of action of the mirror sequence. The sequence might affect the expression or activity of an upstream and common regulator of multiple differentially expressed genes, by competing with normal target binding, similar to mode of action of the sense sequence, described in **Chapter 4**. Therefore, a network analysis was done to construct an interaction map and to identify common regulators of the differentially expressed genes identified with RNA-seq. Optimally, the target of the mirror sequence is the central regulatory gene, for which changes in expression level or activity would explain all other differences in expression level. Although it was impossible to identify one central regulator, different regulators that connect multiple genes from the list of differentially expressed genes could be indicated. These comprise FlhDC, CsgD, GadE, CysB, Cbl, Lrp, H-NS, OmrA and CpxR. Because the mirror sequence is an RNA sequence, it was expected that the activity of an RNA-binding sequence would be reduced in the presence of the mirror sequence and the expression of the targets of this RNA-binding protein would be affected. However, in the list of identified regulators, there are no well-described RNA-binding proteins, although PheNetic is able to map post-transcriptional interactions. Possibly, some of these DNA-binding proteins are able to bind RNA as well. It was indeed suggested that natural sRNAs might also mimic DNA and regulate the activity of DNA-binding proteins (Gottesman & Storz, 2011).

Based on the interaction network, H-NS would also be the best candidate target of the mirror sequence. This DNA-binding protein has indeed been described to bind certain mRNAs (Brescia *et al.*, 2004). Additionally, a reduced activity of this transcriptional regulator would explain why the GadE-based regulatory network is upregulated and FlhDC-based regulatory network is downregulated. However, the binding sequence of H-NS is not identical to the mirror stem loop stem sequence. Therefore, it is also possible that the mirror sequence induces the expression of a compound that reduces the pH and in that way inhibits the expression of H-NS and affects its downstream genes (Soutourina *et al.*, 2002). This would explain why there is no resemblance to the recognition sequence of H-NS. If this is the case, the exact target remains unknown.

Alternatively, based on the identification of the nucleotides in the mirror sequence that are essential for the negative effect on biofilm formation, Lrp was suggested to be another possible target. Because of the similarities between the mirror sequence and the target binding site of Lrp, the sequence is thought to titrate away Lrp. However, for a global regulator, Lrp has a relatively few

interaction partners within the differentially expressed genes, although this is dependent on the number of Lrp targets that are expressed under the conditions tested. Additionally, a reduced activity of Lrp does not explain the large effect on motility genes.

It should be noted that it is possible that some of the gene expression differences identified with RNA-seq are not caused by the mirror sequence, but are growth-dependent gene expression differences between cells expressing the mirror stem loop stem sequence and cells with an empty vector. Although this growth effect is minimized by taking samples at the same OD_{595}, the different growth pattern, i.e. a longer lag phase and a higher OD_{595} in stationary phase, might cause growth phase-dependent differences, which are not directly related to the expression of the mirror stem loop stem sequence. A limitation of the RNA-seq approach is that samples have only been taken at one time point, which gives bias in the results, as it is only gives a picture of the gene expression at a given moment, where only differences in the genes that are expressed at that given moment are measured. However, as we see a difference in the growth pattern in the presence of the mirror sequence, we expected to see some gene expression differences caused by the sequence, which might give a clue why biofilm development is impaired. However, evaluating gene expression at other time points in growth might give more information about the processes that are affected.

Furthermore, it is possible that the mirror sequence has multiple protein targets. Additionally, the sequence might not only be involved in titrating nucleic acid binding proteins, but might also be able to bind RNA molecules and affect the translation efficiency of mRNAs or the regulatory activity of sRNAs. Another effect the sequence might have is that it functions as a decoy for ribosome binding as it resembles an AG-rich Shine Dalgarno region. Although there is also an ATG present, this lies too close to the predicted SD regions to be efficiently bound by the ribosome. However, it is possible that because of this RBS resembling sequence and the presence of an ATG, a small toxic protein is expressed form the plasmid. To identify proteins interacting with the mirror sequence, sRNA-protein complexes can be isolated using aptamer tagged RNAs. The protein partners in the complex can be identified using mass spectrometry (Said *et al.*, 2009).

Finally, the mirror stem loop stem sequence was tested in other strains and species. The sequence negatively affects biofilm formation and growth in another *E. coli* strain, TG1, and in *S.* Typhimurium. In *E. coli* TG1, it requires a longer time before the negative effect on biofilm development can be observed. In *S.* Typhimurium, the reduced amount of biofilm that was observed in the presence of the mirror stem loop stem sequence is thought to be mainly attributed to the reduced growth of the strains. The sequence itself might even improve the biofilm forming capacity of *S.* Typhimurium. This

species-specific effect might be explained by the fact that reducing the activity of the same target can have a different effect on a specific phenotype in another species because the downstream targets of the regulator are different, as is the case for Lrp (Lintner *et al.*, 2008). On the other hand, it can be that the targets of the mirror stem loop stem sequence are different between *S.* Typhimurium and *E. coli*. For example, for FlhDC, the sequence of the 5' leader of the transcript is rather different in *E. coli* and *S.* Typhimurium (Mika & Hengge, 2014). Additionally, the sRNAs that regulate the expression of this regulator are not exactly the same in both species (Campos & Matsumura, 2001).

CHAPTER 7

MicA - *metC*

Studying a specific *in silico* predicted sRNA - mRNA interaction

metC was *in silico* identified as a candidate MicA target in *S.* Typhimurium, based on potential base pair interactions between MicA and the *metC* 5'UTR. However, previous wet-lab experiments suggested that MicA is not directly regulating *metC* mRNA stability or translation efficiency. Therefore, another hypothesis, i.e. that *metC* is regulating MicA stability, was tested here. This would be another example of an mRNA acting as a trap for sRNA activity. However, no significant effect of expressing the *metC* 5'UTR containing the predicted MicA interaction site on MicA stability could be detected.

7.1. Introduction

MicA is a well-studied 70 nt long sRNA encoded on the opposite strand of the intergenic region upstream of *luxS* and downstream of *gshA* (Argaman *et al.*, 2001). Both the sequence of MicA and its genomic organisation are conserved in different Enterobacteriacea (Van Puyvelde *et al.*, 2015), but its biological role was best studied in *E. coli* and *S.* Typhimurium. MicA is shown to be involved in outer membrane remodeling as its expression is controlled by σ^E, the sigma factor that regulates gene expression under envelope stress. Additionally, some of its targets are genes encoding outer membrane proteins, such as *ompA*, *ompX*, *lamB, tsx, ecn* (Bossi & Figueroa-Bossi, 2007; Gogol *et al.*, 2011; Johansen *et al.*, 2008; Rasmussen *et al.*, 2005). MicA reduces the stability of these targets, thereby preventing the presence of misfolded proteins and protecting the cells against envelope stress. However, MicA was shown to have some non-outer membrane targets as well (Coornaert *et al.*, 2010; Gogol *et al.*, 2011).

An *in silico* screen for RNA sequences flanking *S.* Typhimurium translation initiation regions that could base pair to conserved regions within MicA, identified the *S.* Typhimurium *metC* 5'UTR as a high scoring MicA interaction candidate. Therefore, *metC* was predicted to be another target of MicA in this bacterium (Monsieurs, 2006). To validate this prediction, samples were taken shortly after induction of *micA* expression from an inducible promoter. In this short time, only direct regulatory actions have taken place and can be detected based on altered expression levels. Using this technique, it was shown in our lab that induction of *micA* expression caused little effect on *metC* mRNA or protein levels, suggesting that *metC* is not a direct target of MicA (Van Assche, 2010; Van Puyvelde, 2014).

In this chapter we look deeper into another possible regulatory effect of a MicA-*metC* interaction, i.e. the *metC* 5'UTR acting as a trap, which reduces MicA stability. Another sRNA, ChiX, has been shown to be regulated in a similar way in *S.* Typhimurium and in *E. coli* (see **Figure 7.1**) (Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009). ChiX binds to the *chiP* mRNA and consequently reduces *chiP* stability and translation efficiency, unless the polycistronic *chbBCARFG* transcript is present in excess. Then, the *chbB-chbC* intergenic region within this operon binds ChiX, which drastically accelerates the decay of the sRNA by RNaseE because of a destabilization of the terminator loop. This leads to derepression of the *chiP* mRNA. In case of the hypothezised MicA-*metC* trap, *metC* takes up the role of *cbhB-cbhC*.

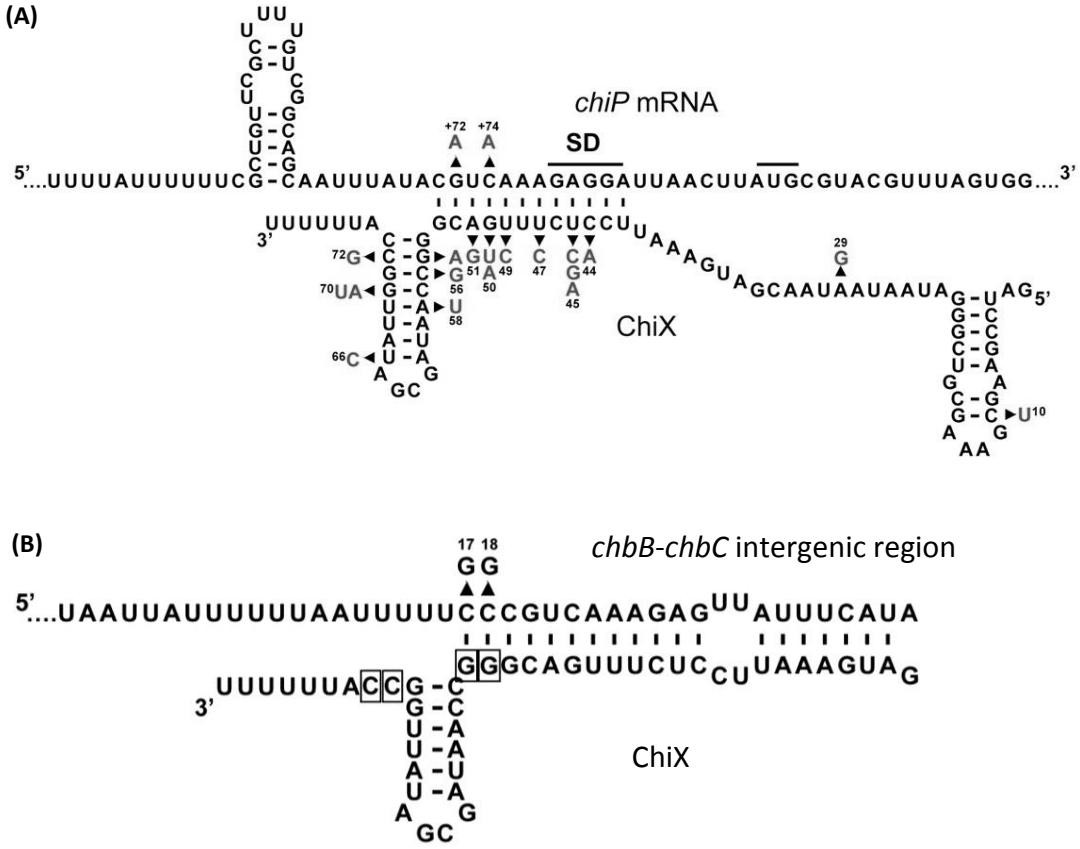


Figure 7.1 (A) Base pairing between ChiX and *chiP* and (B) base pairing between ChiX and the *chbB-chbC* intergenic region. The ChiX region involved in base pairing to the *chbB-chbC* intergenic region involves two bases that are normally involved in the formation of the terminator stem loop of ChiX, which reduces its stability (Figueroa-Bossi *et al.*, 2009).

7.2. Materials and methods

7.2.1 Bacterial strains and growth conditions

Bacteria were routinely grown in LB at 37 °C as described in **section 3.2.1**. The specific strains and plasmids that were used in this chapter are listed in **Table 7.1** and **7.2**, respectively.

Table 7.1 Bacterial strains

Strain name	Description	Reference
***E. coli* Top10**	F´ {*lacIq* Tn10(TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80*lacZΔM15 ΔlacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (*StrR*) *endA1 nupG*	Invitrogen
***S.* Typhimurium SL1344**	*xyl, hisG, rpsL*; virulent; Sm^R	Hoiseth & Stocker, 1981
***S.* Typhimurium CMPG5632**	SL1344 *ΔmetC*	S. De Keersmaecker

Table 7.2 Plasmids

Plasmid name	Description	Reference
pBAD33	Control plasmid for L-arabinose inducible expression from an inducible pBAD promoter, Ap^R	(Papenfort *et al.*, 2006)
pCMPG10940	Plasmid for L-arabinose inducible expression of *metC* with short UTR. The insert was amplified with PRO-532 and S&P-00123 and the vector was amplified with S&P-00124 and S&P-00125.	This work
pCMPG10941	Plasmid for L-arabinose inducible expression of *metC* with long UTR. The insert was amplified with PRO-532 and S&P-00123 and the vector was amplified with S&P-00124 and S&P-00125.	This work

7.2.2 Plasmid construction

The *metC* gene was amplified with PCR using primers containing *XmaI* and *SacI* restriction sites. After digestion of the insert and the vector with these restriction enzymes, the fragments were ligated and transferred to *E. coli* Top10 F' by transformation. Afterwards, the plasmids were isolated and electroporated to the appropriate *S.* Typhimurium strain. All the primers that were used to construct the different plasmids in this chapter, are listed in **Table 7.3**.

Table 7.3 Primers

Primer	Sequence	Purpose	
PRO-532	ATGAGCTCGCATATATGTCCATCCCCGGCAAC	Rv	pCMPG10940 and pCMPG10941,
S&P-00123	ATCCCGGGACTGCCGCACCTTTACTGC	Fw	pCMPG10940
S&P-00124	ATCCCGGGCCAGACGGTTAAAATCAGGAAACG	Fw	pCMPG10941
S&P-00125	ATCCCGGGGGAGAAACAGTAGAGAGTTGC	Fw	pCMPG10940 and pCMPG10941
S&P-00126	ATTGAGCTCTTAAATCAGAACGCAGAAGC	Rv	pCMPG10940 and pCMPG10941

7.2.3 RNA isolation, northern blot analysis and qRT-PCR analysis

RNA was isolated as described in **section 3.2.3**. Northern blot analysis was done as described in **section 3.2.4**, only, here, the RNA was separated on a 6 % polyacrylamide / 6 M ureum gel and the probes, which are listed in **Table 7.4**, were hybridized at 42 °C. cDNA was made as described in **section 3.2.5**. The primers for qRT-PCR analysis that are specifically used in this chapter are listed in **Table 7.5**.

Table 7.4 DIG-labeled probes for northern blot detection

Probe	Sequence	Target	Reference
PRO-0548	GATAACAAATGCGCGTC	MicA	(Urban & Vogel, 2007)
PRO-0157	CTACGGCGTTTCACTTCTGAGTTC	5S rRNA (loading control)	(Papenfort *et al.*, 2006)

Table 7.5 qRT-PCR primers

Primer	Sequence	Purpose		Reference
pro-2527	CCTACGGCGCTGACAACTTTA	Rv	ompA	(Papenfort *et al.*, 2006)
pro-2581	CTTCATTCACAATGATGGCCC	Fw	ompA	(Papenfort *et al.*, 2006)
PRO-3514	GGGAACGCTGACCCACTTT	Fw	metC	Van Puyvelde, 2014
PRO-3515	CCGCCTTCCAGTTCACACAT	Rv	metC	Van Puyvelde, 2014
PRO-7841	AGTTCCAGTGTGGCTGGTCAT	Fw	rrnD	(Jonas et al., 2008)
PRO-7842	GCTCACCAAGGCGACGAT	Rv	rrnD	(Jonas et al., 2008)
PRO-8243	CGCATTAAAAAAGCCACCTGTT	Fw	metCUTR	This work
PRO-8244	ACCCTGGCGACGTGTGA	Rv	metCUTR	This work

7.3. Results

The hypothesis that *metC* acts as an sRNA trap, sequestering MicA and consequently reducing its stability upon binding, is based on the alternative MicA region that is predicted to interact with the *metC* 5' UTR. Based on complementary base pairing, all of the validated MicA targets identified today are shown to interact with block 1 of MicA (see **Figure 7.2 (A)**), while MicA is predicted to interact with the *metC* 5' UTR with block 2 (see **Figure 7.2 (A)** and **(B)**). Part of this block 2 region is important for the formation of the terminator loop, which is known to be important for MicA stability (Andrade *et al.*, 2013). Therefore, the interaction of the *metC* 5' UTR and MicA might break the terminator loop

structure, reducing sRNA stability, similarly to what has been described for ChiX and *cbhB-cbhC* (see **Figure 7.1**).

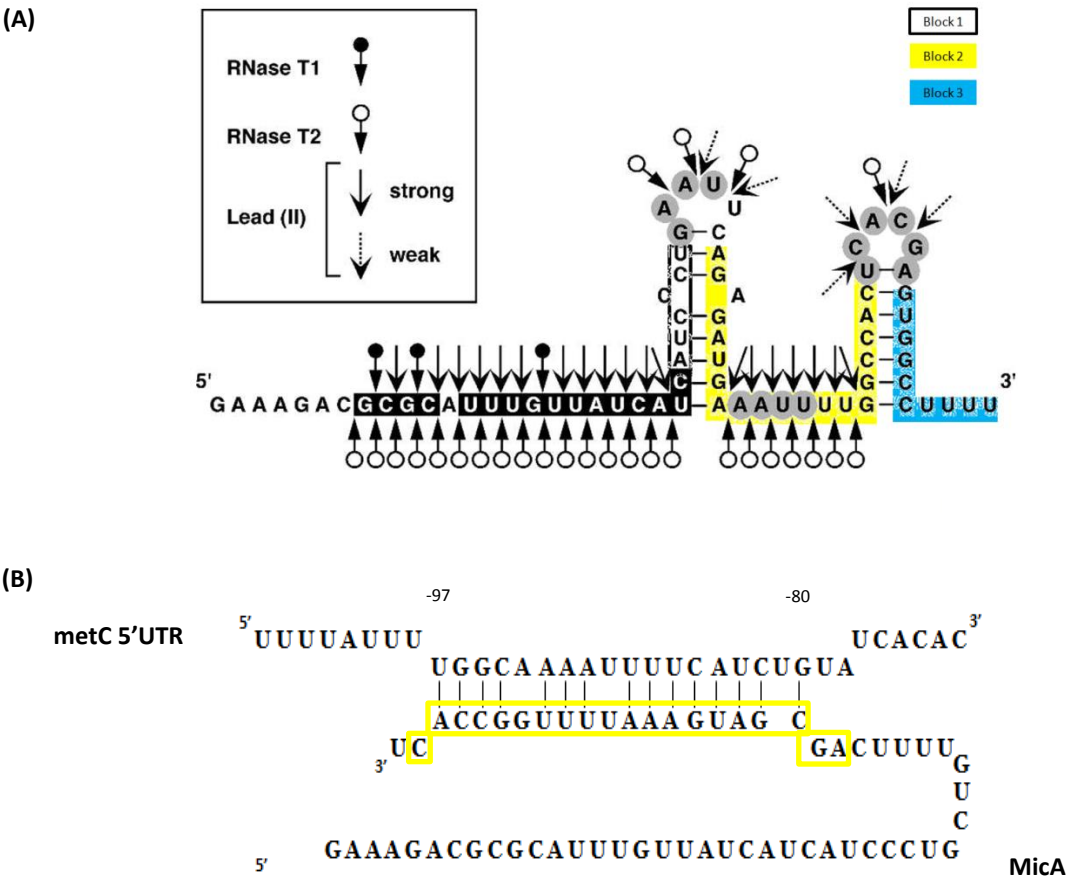


Figure 7.2 (A) Predicted secondary structure of MicA (adapted from Udekwu et al., 2005). The structure consists of a single stranded region at the 5' end, a structured region harbouring two stem loops, separated by an A/U-rich region and the transcriptional termination U-rich sequence. The different colors indicate the different blocks within the MicA sequence that are conserved among closely related Enterobacteriaceae (De Keersmaecker *et al.*, 2006). **(B) Predicted interaction between MicA and the *metC* 5' UTR.** The MicA nucleotides in yellow correspond to the nucleotides of block 2 in **(A)**.

7.3.1 Effect of *metC* overexpression on *micA* expression

To validate the hypothesis that *metC* affects MicA stability, *metC* expression was induced from an arabinose inducible pBAD promoter in a *ΔmetC* strain and consequently, the effect on *micA* expression was tested. If the *metC* 5'UTR reduces MicA stability, a reduction in MicA expression levels should be observed (Papenfort *et al.*, 2006; Sharma & Vogel, 2009). Both *metC* with a long

5'UTR, which includes the region predicted to interact with MicA (pCMPG10940), and *metC* with a shorter 5'UTR, *i.e.* without the predicted MicA interaction region (pCMPG10941), were induced (for a schematic overview, see **Figure 7.3**). Additionally, a control plasmid, which is an empty pBAD vector, was included in the test.

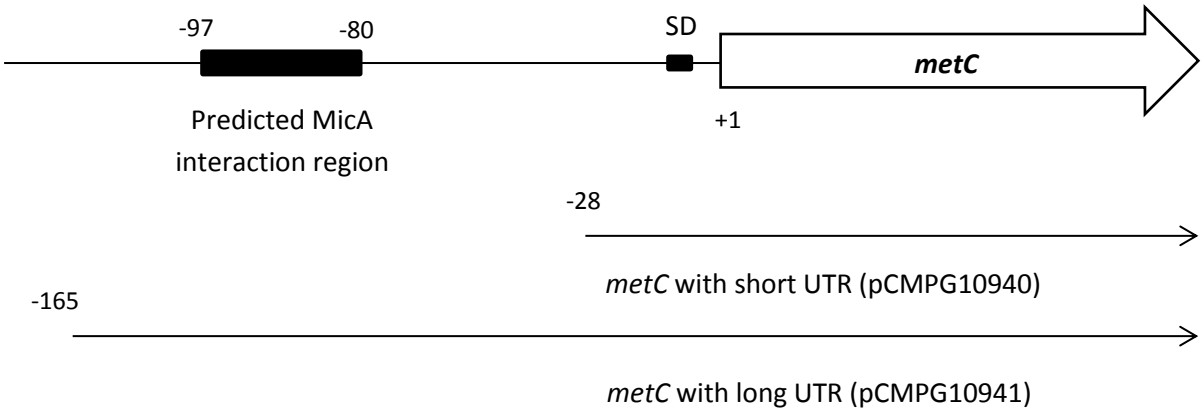


Figure 7.3 Schematic representation of the *metC* transcripts with a short and a long UTR. The transcript with the short UTR does not include the predicted MicA interaction region, contrary to the transcript with the long UTR. The positions indicated are relative to the *metC* startcodon. SD represents the Shine-Dalgarno region.

In **Figure 7.4** the *metC* expression levels are shown for the control strain and for the strains with a plasmid encoding *metC* with a long and a short UTR, for samples taken before and 10 minutes after induction with arabinose. These expression levels are both measured using primers targeting the *metC* coding region (see **Figure 7.4 (A)**) and primers targeting the *metC* 5' UTR in the region surrounding the predicted MicA interaction region (see **Figure 7.4 (B)**). The results show that by inducing the expression of *metC* with arabinose, *metC* expression levels rise drastically. The levels of the coding region indeed increase both when *metC* with the long UTR and when *metC* with the short UTR is induced (**Figure 7.4 (A)**). When using primers targeting the *metC* 5' UTR, in the region surrounding the predicted MicA interaction site, the rise in expression is only detected for strains with a plasmid encoding *metC* with the long UTR, as was expected.

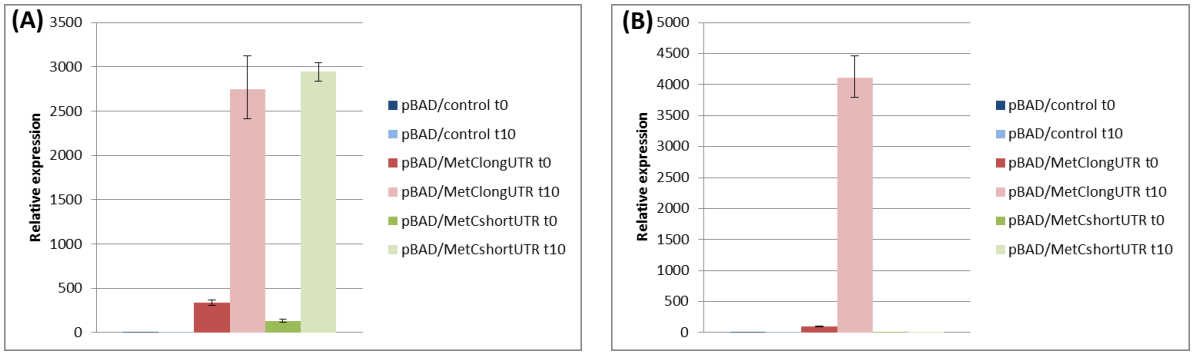


Figure 7.4 qRT-PCR results showing the *metC* expression levels after *metC* pulse expression (A) using primers targeting the *metC* coding region and (B) using primers targeting the *metC* 5'UTR region surrounding the predicted MicA interaction region. The RNA samples are taken from *S.* Typhimurium Δ*metC* strains with a control plasmid, with a plasmid expressing *metC* with a long UTR (pCMPG10941) and with a plasmid expressing *metC* with a short UTR (pCMPG10940). The strains were grown in LB at 37 °C until OD 2. Then, the *metC* expression was induced with 0.02 % arabinose and samples were taken just before and 10 minutes after induction. Error bars represent 95 % confidence intervals.

Consequently, the effect of *metC* induction on the *micA* expression levels was evaluated using northern blot analysis. Hereto, 4 µg of total RNA of each RNA sample was loaded on the gel. MicA RNA levels were detected using a specific probe (PRO-0548). Detection with the 5S rRNA probe (PRO-0157) was included as loading control. The results are shown in **Figure 7.5**.

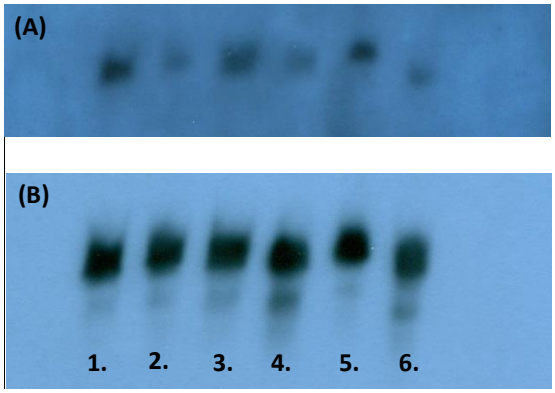


Figure 7.5 Northern blot analysis of *micA* expression after pulse expression of *metC* with different UTR lengths (A) using a MicA specific probe (PRO-0548) and **(B)** using probe targeting 5S ribosomal RNA (PRO-0157). **1.** pBAD/control before induction, **2.** pBAD/control 10 minutes after induction, **3.** pBAD/*metC* long UTR before induction, **4.** pBAD/*metC* long UTR 10 minutes after induction, **5.** pBAD/*metC* short UTR before

induction, **6.** pBAD/*metC* short UTR 10 minutes after induction. Exposure times are not equal between panel (A) and (B).

The results in **Figure 7.5 (A)** show that *micA* expression is reduced after the induction of *metC* from an arabinose inducible promoter. However, this observation can be made in all three strains, also in the strain with the control plasmid, suggesting that it is the arabinose that has a negative effect on *micA* expression levels. Nevertheless, taking into account the loading control (**Figure 7.5 (B)**), which suggests that more total RNA is loaded of the RNA sample of cells after the induction of *metC* with a long UTR (sample 4), the results could suggest that *micA* expression levels are more reduced in this sample, compared to the samples of cells where *metC* with a short UTR is induced or cells with the control plasmid after induction with arabinose.

7.3.2 Effect of *metC* overexpression on *ompA* expression

As it is difficult to quantify expression levels using northern blot analysis, qRT-PCR analyses are preferred. However, it is very hard to detect MicA levels with qRT-PCR, as this sRNA is encoded on the opposite strand of *luxS* (Kint *et al.*, 2010), with both transcripts overlapping. When a double stranded cDNA library is made, it is impossible to distinguish *luxS* and *micA* transcripts. Therefore, the mRNA levels of *ompA* were measured. *ompA* is the first described target of MicA. *micA* expression reduces *ompA* expression levels by binding with block 1 near the ribosome binding site of *ompA*. Therefore, changes in expression levels of *ompA* are an indirect measure of changes in the MicA levels. The *ompA* expression levels in the samples after induction of *metC* are given in **Figure 7.6**.

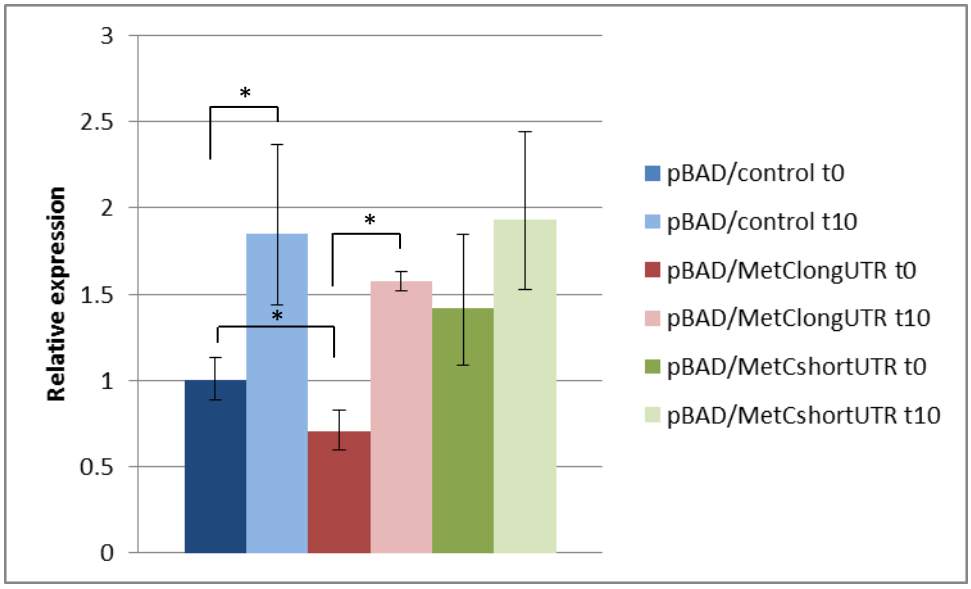


Figure 7.6 qRT-PCT results showing *ompA* expression levels after *metC* pulse expression. The RNA samples are taken from *S.* Typhimurium Δ*metC* strains with a control plasmid, with a plasmid expressing *metC* with a long UTR (pCMPG10941) and with a plasmid expressing *metC* with a short UTR (pCMPG10940). The strains were grown in LB at 37 °C until OD 2. Then, the *metC* expression was induced with 0.02 % of arabinose and samples were taken just before and 10 minutes after induction. Error bars represent 95 % confidence intervals. A star indicates that the C_T values are significantly different for the different samples ($p < 0.05$).

The results in **Figure 7.6** show that after the addition of arabinose, expression of *ompA* increases. As northern blot analyses (see **Figure 7.5**) showed that MicA levels drop after the addition of arabinose, this confirms that there is indeed a negative correlation between MicA and *ompA* expression levels. However, there is no difference in *ompA* expression levels between the samples taken after induction of *metC* expression with a long or short UTR, suggesting that there is no difference in MicA expression levels between these samples either. The increase in *ompA* expression levels is slightly higher for the strain expressing *metC* with a long UTR if the samples before and after induction are compared, suggesting a larger decrease in MicA expression. However, the difference is small. Consequently, it can be concluded that the presence of the predicted MicA interaction site within the *metC* 5' UTR has a limited to no effect on MicA stability.

7.4. Discussion

MicA is an sRNA that has already been studied for more than ten years. During this time, quite a number of different MicA targets have been identified (Bossi & Figueroa-Bossi, 2007; Coornaert *et al.*, 2010; Gogol *et al.*, 2011; Johansen *et al.*, 2008; Rasmussen *et al.*, 2005). In *S.* Typhimurium, *metC* was predicted *in silico* to be one of these targets (Monsieurs, 2006). However, this prediction could not be validated in wet-lab experiments (Van Puyvelde, 2014). Nevertheless, given that the predicted interaction site within the *metC* 5' UTR is relatively far upstream of the *metC* start codon and part of the predicted interaction site within MicA is important for the formation of the terminator stem loop (Andrade *et al.*, 2013), another possible regulatory effect of this interaction was studied. Indeed, *metC* might be a trap for the sRNA MicA, thereby reducing MicA stability without affecting its own. This mode of action would not be unique to MicA. The stability of another sRNA, ChiX, has indeed been described to be regulated in a similar way, both in *E. coli* and in *S.* Typhimurium (Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009). Additionally, in eukaryotes, transcripts sequestering miRNAs, the eukaryotic counterparts of sRNAs, through decoy sites have also been identified (Banks *et al.*, 2012; de Giorgio *et al.*, 2013; Kartha & Subramanian, 2014). It was moreover suggested that mRNAs that can act as sponges for sRNA sequestration have been neglected so far, but might be of great importance (Göpel & Görke, 2014).

MicA levels were measured both directly and indirectly, by measuring the expression of a MicA target, *ompA.* No significant difference in *micA* expression levels could be observed between strains that express *metC* with or without the predicted MicA interaction site in their 5' UTR. This result suggests that the predicted MicA interaction region within *metC* does not influence MicA stability. Additionally, an immunoprecipitation experiment with Hfq did not identify *metC* as being bound to Hfq, suggesting that it is not susceptible to Hfq dependent sRNA-mediated regulation (Chao *et al.*, 2012). Although *ompA*, known to be regulated by sRNAs was also not retained in this list and Hfq-independent sRNA have been identified, this reduces the likelihood of *metC* being a sRNA interaction partner.

CHAPTER 8

Conclusions

General discussion and conclusions

In this final chapter, the general conclusions of this PhD research are formulated and commented. Additionally, some future perspectives regarding nucleic acid-based biofilm inhibitors are given. Overall, the experiments in this PhD thesis show that nucleic acid sequences are a novel and promising type of biofilm inhibitor. However, the application of the identified small sense sequence as a anti-biofilm molecule needs further optimization regarding the nucleic acid analog, linker or CPP used to increase its potency. Additionally, testing in more 'real life' environments, on natural isolates and on multispecies biofilms that contain an *E. coli* fraction is required. The mirror sequence showed that other biofilm inhibitory sequences can be identified. More specifically, other sRNA regulated RNA-binding proteins might be interesting targets and interfering with their activity by applying the same approach as described here is interesting for further study. Additionally, the mirror sequence and sequences that target DNA-binding proteins or sRNA regulators of biofilm development, for which increasing knowledge becomes available, might in the future be used as a biofilm inhibitor as well.

8.1. General discussion

Biofilms are the most common mode of growth of bacteria in nature. Bacteria in a biofilm have an increased tolerance against antibiotics, disinfectants and components of the immune system (Høiby *et al.*, 2010). Therefore, detrimental biofilms, for instance in industrial or medical settings, are difficult to remove or treat which results in significant economic losses and medical threats (Van Houdt & Michiels, 2010). Hence, there is a need for molecules that prevent biofilm formation, which can be used alone or in combination with conventional antimicrobial approaches. The increasing knowledge about the different processes and cellular components involved in biofilm development enables the rational design of such anti-biofilm molecules. For example, it has already been shown that attachment can be prevented and matrix stability can be disturbed based on the knowledge about adhesion factors and the composition of the matrix, respectively (Guiton *et al.*, 2012; Hymes *et al.*, 2013; Kaplan *et al.*, 2004; Okshevsky *et al.*, 2015; Pleszczyńska *et al.*, 2015; Totsika *et al.*, 2013). An alternative anti-biofilm approach is to specifically interfere with the expression or the activity of known regulators of biofilm development, such as quorum sensing molecules, c-di-GMP and sRNAs (Wolska *et al.*, 2015).

Here, we focused on the rational design of sequences that interfere with the expression or activity of sRNAs (see **Figure 8.1**), which are recently identified as important biofilm regulators (Martínez & Vadyvaloo, 2014). To the best of our knowledge, this is a biofilm inhibitory strategy that has not been described before. However, RNA molecules can be useful targets to interfere with, as they can be mimicked or targeted with nucleic acids or their synthetic analogs. Moreover, sRNAs have a small and well-described target interaction region that can be sufficient for their function. Targeting or mimicking this small region avoids difficulties in the uptake of larger molecules.

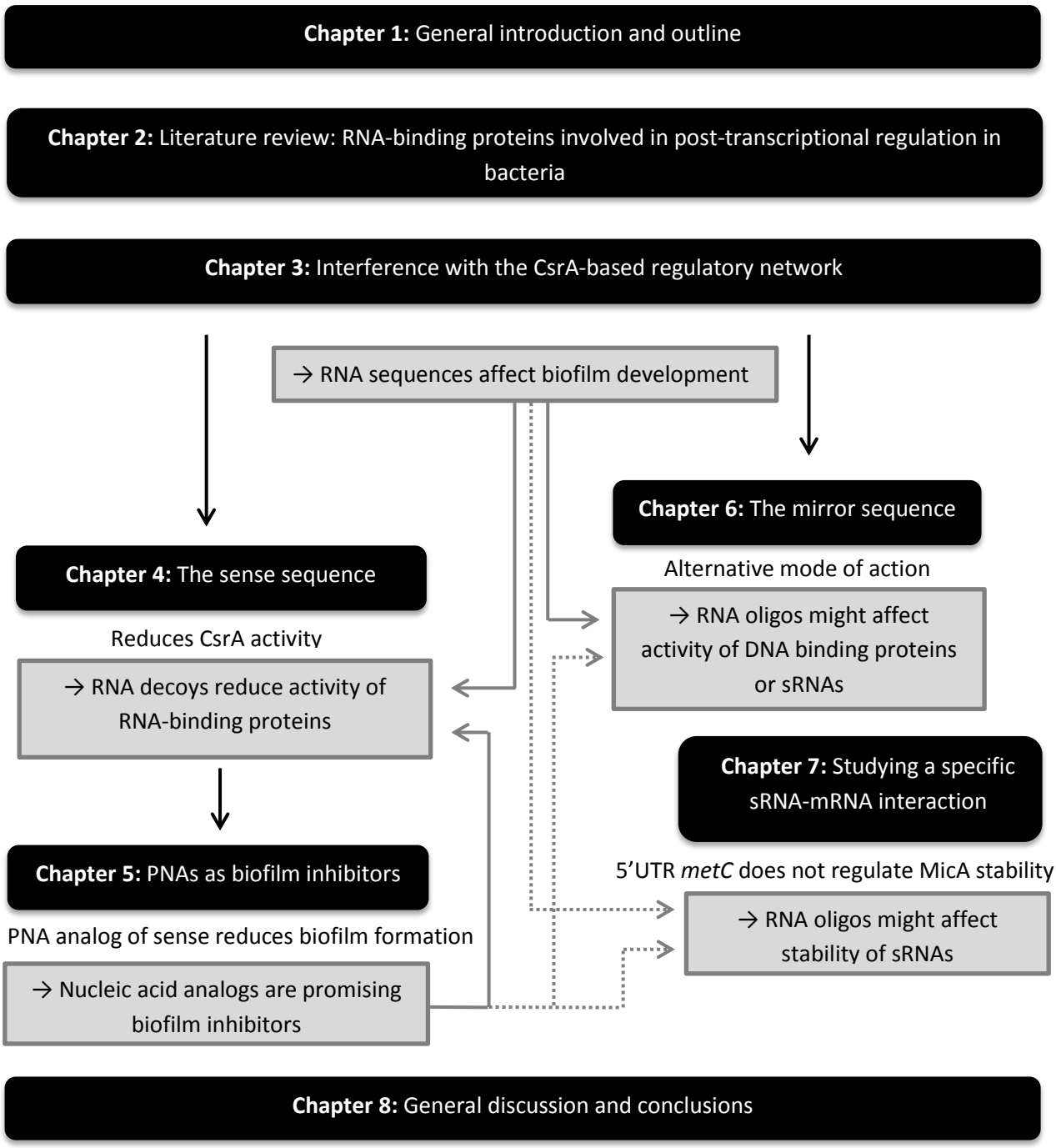


Figure 8.1 Overview of the study. Arrows in a full line represent a connection that was established in this work. Arrows in a dotted line represent possible connections that were not yet validated but might be interesting for future research.

8.1.1 Can natural or chemically modified nucleic acid sequences which target or mimic a target interaction site of a sRNA be used as a biofilm inhibitor?

Our hypothesis that nucleic acid sequences, targeting or mimicking a target interaction site of a sRNA, can be used as a biofilm inhibitor, was tested using the CsrA-based regulatory network. This regulatory system is known to be involved in the regulation of biofilm development and includes sRNA-based regulation through, amongst others, CsrB (Jackson *et al.*, 2002). Based on the knowledge about the selectivity of CsrA and the nucleotides within CsrB that are important for interaction with CsrA, it was possible to rationally design small antisense and sense sequences that mimic or target CsrB, which might interfere with CsrA or CsrB activity and impair biofilm development.

For the antisense approach, the experiments in **Chapter 3** showed that neither the complete *csrB* sequence in antisense nor the small antisense sequences based on the target interaction site of the sRNA affect biofilm development under the conditions tested. One reason for this might be the extensive secondary structure of CsrB that prevents its interaction with other RNA molecules. Another option is that other sRNAs with the same function, like CsrC, take over the role of CsrB. Possibly RNA-binding sRNA are more vulnerable than protein binding sRNAs for this type of interference, as their target interaction region might be more accessible for RNA-RNA interactions. Therefore, this antisense strategy can be interesting for further study using other sRNAs involved in biofilm development.

The small sense stem loop stem sequence, on the other hand, was able to reduce biofilm formation of *E. coli* DH5α, just like the complete CsrB sequence in sense. These results confirmed that short natural nucleic acid sequences can reduce the biofilm forming ability of a bacterial strain. Subsequently, the mode of action of this interesting sequence was validated in **Chapter 4**. The sense stem loop stem sequence was designed to mimic the target interaction region of CsrB, thereby binding to CsrA and reducing CsrA activity through sequestration. A gene expression study and a mutational analysis confirmed that the sense stem loop stem sequence acts in this way and showed that nucleic acid sequences can be used to interfere with the activity of biofilm regulators and that the CsrA/CsrB system is interesting for this kind of application.

A synthetic PNA sequence coupled to a $(KFF)_3K$ CPP that is based on the sequence of the sense stem loop sequence was subsequently tested in **Chapter 5** as a biofilm inhibitor that was externally added to the growth medium. Our experiments showed that biofilm formation of *E. coli* DH5α in the

presence of the CPP-coupled PNA based on the sense stem loop stem sequence was reduced in a sequence-specific manner. However, a gene expression analysis showed that the expression pattern of the CsrA targets tested is not similarly affected in strains grown in the presence of the PNA and in strains that express the sequence from a plasmid. Consequently, it could not be confirmed yet that the PNA and the plasmid-expressed sense stem loop stem sequence have the same mode of action. Probably, a higher concentration of the PNA is necessary to observe an effect on the expression of these targets.

Although these results thus suggest that mimicking the target interaction region of CsrB can be an interesting biofilm reducing strategy, there are possibilities to improve the efficiency. The plasmid expressed small sequence reduces biofilm formation to a lesser extent than the full length *csrB* sequence in sense. The efficiency of the small sequence might therefore be enhanced, possibly by coupling two or more of the small target interaction sequences, just like in CsrB, or by coupling stabilizing elements. Because of size constrains for the uptake of PNAs through the cell membrane, the potency of the synthetic sequences cannot be increased by enlarging the sequences, but might benefit from an optimization of the type of nucleic acid analog, the linker or the CPP that is used. Regarding the CPP, the use of a different cell penetrating peptide, like the HIV-based sequence would most likely reduce the toxicity and non-sequence specific effect of the PNA and is less susceptible to resistance development. Regarding the type of nucleic acid analog, PNAs are not more efficient tools compared to PMOs, LNAs or other analogs. However, they were an interesting option as they can sequence-specific and efficiently hybridize DNA and RNA, can perform strand invasion and pre-clinical experimental models demonstrate that PNA-based molecules are able to alter gene expression *in vivo*. Other nucleic acid analogs can therefore be tested, but hybrids of PNA/RNA are assumed to be the most interesting alternatives for this sequence. If the sequence still needs to be enlarged after optimization of the CPP and the nucleic acid analog to have an optimal effect, other modes of internalization in the cell would be required. However, at this moment, CPP-based delivery still outcompetes other delivery strategies. For the PNAs that were tested here, the effective concentration is already comparable to the concentration of other identified biofilm inhibitors (Steenackers *et al.*, 2011).

Remarkably, our results suggested a condition-dependent effect of CsrB on biofilm development. This conditionality might limit the applications in which this sequence can be used and therefore requires further study. Since biofilms formed under different conditions, like different temperatures or growth media, generally have a different composition, this condition dependency is an important

point to take into account, not only for this CsrB mimicking sequence, but for all biofilm inhibitors that are developed.

Besides the condition dependency, biofilm tests in *E. coli* TG1 and in *S.* Typhimurium demonstrated that the effect of expressing the sense stem loop stem sequence on biofilm development is species-specific. The same species-selectivity was observed in strains grown in the presence of the PNA. Earlier studies indeed showed that CPP-PNAs can indeed be used to reduce the growth of bacteria in a species-specific manner (Mondhe *et al.*, 2014). Because of this specificity, a combination of different nucleic acid sequences might be necessary if different species cause problems in a specific setting. Additionally, their application requires knowledge about the composition of the microbial community. On the positive side, a highly specific sequence can remove pathogenic bacteria, but safeguard harmless or beneficial bacteria (Mondhe *et al.*, 2014).

8.1.2 Are there other interesting biofilm inhibitory nucleic acid sequences?

Another interesting sequence identified in the course of this work, is the mirror stem loop stem sequence. This sequence has a strong negative effect on biofilm formation and growth of *E. coli* DH5α. Additionally, the sequence was shown to reduce biofilm formation in *E. coli* TG1 and in *S.* Typhimurium as well, although species-specific differences were observed. Therefore, it might be interesting to make a synthetic sequence based on this mirror sequence and test its anti-biofilm activity. However, the negative effect of the mirror sequence on the growth of the bacteria might also be a factor increasing the risk for resistance development against this sequence, as a reduction in survival increases the selection pressure for resistance development.

Although the mirror sequence was shown to have a strong negative effect on biofilm development and growth, the mode of action of the sequence is unclear (see **Chapter 6**). The mirror sequence slightly resembles a CsrA binding site. However, the analysis of the expression levels of the CsrA targets showed that the sequence does not affect CsrA activity. A subsequent transcriptome analysis showed that both flagella and curli production was downregulated in the presence of the mirror stem loop stem sequence, which are both important for biofilm development. Additionally, pH-regulated genes were upregulated, suggesting that the target is involved in the regulation of genes induced at low pH or the sequence causes pH-stress, which might explain the reduced growth. Because of the importance of the secondary structure of the sequence for the anti-biofilm effect, regulatory proteins are more likely to be the interaction partners of the sequence, as RNA-RNA

interactions would require single stranded accessible regions for efficient base pairing. However, the secondary structure might also be important for the stability of the RNA molecule itself. A network analysis enabled the identification of a few central regulators, for which a deregulation might explain the observed gene expression differences, such as H-NS and Lrp. For H-NS, it has been shown that this protein plays an essential role in biofilm formation in *E. coli* (Belik *et al.*, 2009). Therefore, interference with its activity can explain the reducing effect on biofilm development. However, as both H-NS and Lrp are global regulators, their identification as a true target requires further validation. Alternatively, as the sequence resembles a ribosome binding site, it might be that there is a general interference with transcription. Possibly, the sequence has multiple targets as well, which do not only involve nucleic acid binding proteins, but also sRNAs or mRNAs, affecting their activity or expression. Protein partners of the sequence can be identified by isolating the RNA/protein complex using aptamer tagged sequences, after which the proteins can be identified using mass spectrometry. Alternatively, a mutant library can be screened for genes that abolish the effect on growth and biofilm development, which might give a clue about the target.

Besides the mirror sequence, other biofilm inhibitory sequences can be tested. The experiments in this work indeed show that biofilm regulating RNA-binding proteins can be interesting targets for anti-biofilm components and that nucleic acid sequences can be used to interfere with their activity. Therefore, these results suggest that other protein regulating sRNAs and their targets might also be valuable targets for nucleic acid-based biofilm inhibitors. Specifically Hfq seems of special interest. This protein is a global post-transcriptional regulator that is necessary for most sRNA-dependent regulatory interactions (Sauer, 2013b). The activity of this protein in *Pseudomonas aeruginosa* was recently shown to be similarly regulated as CsrA, with an sRNA that mimics the preferred RNA sequence for binding (Sonnleitner & Bläsi, 2014). Therefore, similar strategies might be applied to interfere with the regulatory activity of this protein and might disturb the processes that are controlled by this regulator. Also the activity of RapZ is regulated by a sRNA (Göpel *et al.*, 2013). However, this protein was not shown to be involved in biofilm development.

Additionally, as many RNA-binding sRNAs are also involved in biofilm development, it might be interesting to explore the possibilities of designing sequences that reduce the activity of these sRNAs. Although our attempts to hinder the activity of the sRNA CsrB with antisense sequences were unsuccessful, other RNA-binding sRNAs, like OmrA, OmrB, McaS, RprA, which regulate *csgD* expression, might be more suitable for an antisense approach, targeting the region in the sRNA that is important for target interaction. Additionally, as more regulatory mechanisms applied by natural RNAs are described, such as the regulation of sRNA stability with mRNA molecules, these might be an

interesting source of inspiration for alternative approaches to disturb the regulatory actions of specific sRNAs involved in biofilm development. Regarding this alternative regulatory mechanisms, an *in silico* predicted sRNA-mRNA interaction between MicA and *metC* was studied in **Chapter 7**. It was hypothesized that *metC* regulates MicA stability, based on similarities with the *cbhBC*-ChiX interaction, described in literature. However, the experiments done here did not confirm such a regulatory role for *metC*. Still, this mode of action might be an inspiration for future RNA-based biofilm inhibitors targeting sRNAs.

8.1.3 Are chemically modified nucleic acid analogs the antimicrobials of the future?

Our results suggest that CPP-coupled nucleic acid sequences are interesting anti-biofilm molecules. Their use as biofilm inhibitors is new, but they were already studied for their use in other antimicrobial applications. Indeed, sequences targeting the translation start site of essential genes were shown to reduce the growth of bacteria, to decrease infection in a mouse model and to increase survival of these mice, not only for *E. coli*, but also for *Burkholderia* infections (Greenberg *et al.*, 2010; Tan *et al.*, 2005; Tilley *et al.*, 2007). Additionally, they were shown to inhibit intracellular growth of *Salmonella* in tissue culture (Mitev *et al.*, 2009). With MIC values in low micromolar range, these nucleic acid analog sequences can compete with the currently used antibiotics and are therefore very promising.

However, with every new antimicrobial or anti-biofilm compound identified, the question of resistance against this compound arises. Developing resistance against molecules that reduce the activity of an RNA-binding protein using decoys is assumed to be rare, as this would require mutations in the target interaction site of CsrA and then, interaction with its mRNA targets would be affected as well. This would disturb the complete regulatory network and is therefore unlikely. Moreover, PNAs have been shown not to be substrates for efflux pumps (Nikravesh *et al.*, 2007). However, resistance can also be developed against the coupled CPP, necessary for the uptake of the nucleic acid analog. Two studies specifically tested the resistance against an antisense CPP-coupled PNA or CPP-coupled PMO, another nucleic acid analog (Ghosal *et al.*, 2012; Puckett *et al.*, 2012). The authors showed that resistance of a spontaneous mutant could be linked to *sbmA*, a gene that is involved in the transport of the CPP-PMO or CPP-PNA across the membrane. Therefore, an optimization of the strategies applied for the uptake of the sequences into the cell is still needed. However, some natural antimicrobial peptides, which have characteristics similar to the synthetic cell

penetrating peptides used, are already being developed and do not appear to cause resistance in bacteria (Splith & Neundorf, 2011). Additionally, alternative delivery strategies, using liposomes or nano-particles can be interesting.

For their use as an antimicrobial, additional testing regarding toxicity, bioavailability and pharmacokinetics has been suggested (Bai & Luo, 2012). The same tests might also be required for anti-biofilm nucleic acid sequences. However, this depends on the specific application. Since in our tests, the effect of the CPP-PNA on biofilm formation was shown to be effective in poor medium, under ambient conditions, their application will probably be in industrial settings, for instance as a slow release coating on surfaces. In this case, bioavailability and pharmacokinetic studies are less relevant. Alternatively, their use in these industrial settings requires cheap production. The PNAs used in this study are expensive, but their production in bulk might reduce the production cost. For other biofilm inhibitory sequences that will be used in humans, although not yet identified, pharmacological tests will probably be required.

8.1.4 Are chemically modified nucleic acid analogs promising for other therapies?

Besides in antimicrobial applications, nucleic acid sequences and their analogs have also proven their applicability in the treatment of genetic or viral diseases (McClorey & Wood, 2015). Chemically modified nucleic acid sequences have already been used to target or mimic miRNAs, the eukaryotic counterpart of bacterial sRNAs. For example, both phosphorothioate modified sequences and a LNA/DNA hybrid have been shown to target and reduce miR-122 expression and were evaluated in a phase II clinical trial to inhibit hepatitis C virus (Janssen *et al.*, 2013; Krützfeldt *et al.*, 2005). Another LNA-DNA hybrid is able to bind and inhibit the miR-34 family and reduces cardiac remodeling and arterial enlargement in myocardial infection (Bernardo *et al.*, 2012). Also microRNA mimics have gone into phase I clinical trials for cancer treatment (Bouchie, 2013). Other miRNA targeting or miRNA mimicking sequences involved in anticancer treatment have been identified, but are often in preclinical stages of development (McClorey & Wood, 2015).

Additionally, antisense oligonucleotides targeting mRNAs are also used for certain therapies. One example is an antisense oligonucleotide active in downregulating transthyretin, of which elevated levels are responsible for familial amyloid polyneuropathy or familial amyloidotic cardiomyopathy (Coelho *et al.*, 2013). This RNA oligo is in phase III clinical trial for the treatment of these diseases.

Another example is the use of a hybrid of DNA and 2'-O-methoxyethyl modified nucleic acids targeting the *apoB* mRNA, which have been tested as an adjuvant therapy in familial hypercholesterolemia (Raal *et al.*, 2010).

All these examples illustrate that many nucleic acid analog sequences targeting regulatory RNAs or mRNAs are in the pipeline as novel treatment options. However, despite the fact that *in vivo* data on PNA treatments for genetic or infectious diseases are also available (Fabani *et al.*, 2010; Tan *et al.*, 2005), no clinical trials have been activated for PNA based therapeutics, but are expected for the near future (Gambari, 2014).

8.2. Conclusion

The experiments described here show that nucleic acid sequences can be interesting anti-biofilm molecules. More specifically, it was demonstrated that the rational design of RNA sequences that interfere with the activity of CsrA, a post-transcriptionally active RNA-binding protein involved in the regulation of biofilm development, is a promising novel anti-biofilm strategy. Moreover, a PNA-based synthetic analog of the sense stem loop stem sequence was shown to reduce biofilm formation in a sequence-specific manner, which looks promising for the development of a biofilm inhibitor. However, its application requires further testing in more complex model systems and 'real life' environments and further optimization regarding the nucleic acid analog, the CPP or the linker used. Based on the increasing effort that has been put in the development of nucleic acid analogs and CPPs with better characteristics, driven by antimicrobial, but also antiviral and anticancer research, its application as an anti-biofilm compound can benefit from the knowledge in these fields and their characteristics and potency can be improved relatively easy. An element that requires specific attention is the condition dependency of the CsrB-CsrA based system as it determines the application in which the sequence can be used. Moreover, as our tests show that the sense sequence is only active in reducing *E. coli* biofilms, the development of other biofilm inhibitory sequences is necessary for other bacteria that cause problems. Therefore, it is interesting to study the possibilities of targeting other RNA-binding post-transcriptional regulators, like Hfq, using the same approach. Alternatively, the use of nucleic acid sequences targeting DNA-binding proteins, sRNAs or mRNAs as biofilm inhibitors, is another interesting topic for further study.

In conclusion, the potency of nucleic acid analog sequences as anti-biofilm molecules is until now underestimated, but deserves more attention as it is a relatively easy way to target the genes and regulators that have been identified as being important for biofilm development. Although our work is only the start of the development of such sequences, it demonstrates that nucleic acid sequences targeting biofilm regulators are a novel and promising type of biofilm inhibitor.

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