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Engineering Bacteria to Disperse Bacterial Biofilms

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Dedicated to my parents

तीर्थरूप आई आणि बाबा यांना समर्पित

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Zusammenfassung

Biofilme sind Aggregate von Bakterien, welche in einer selbst produzierten Matrix eingebettet sind. In der Natur existieren die meisten Bakterien in Form von Biofilmen. Diese sind außergewöhnlich resistent gegen Umweltstress. Sie können raue Umweltbedingungen wie Hunger und Austrocknung überleben und herkömmlichen antimikrobiellen Substanzen widerstehen. Bakterien in Form von Biofilmen verursachen eine Reihe von Krankheiten in Menschen, Tieren und Pflanzen. Die Bildung von Biofilmen führt zu Korrosion von Rohrleitungen und verursacht immense Verluste in der Industrie. Der Hauptfokus der mikrobiologischen Forschung verlagert sich zunehmend in Richtung der Biofilme, wobei Forscher Strategien zur Bekämpfung von Biofilmen untersuchen.

Unter den verschiedenen Strategien zur Bekämpfung von Biofilmen besitzen biologische Methoden einige Vorteile gegenber anderen Methoden. Biologische Methoden nutzen bereits existierende Signalwege in Bakterien aus und bewirken eine effiziente Zersetzung des Zielbiofilms ohne die Verwendung von künstlich synthetisierten Substanzen. Solche Methoden sind insbesondere attraktiv für die Behandlung von Biofilmen, die Krankheiten verursachen. In diesem Projekt habe ich drei verschiedene Strategien ergründet, um Escherichia coli für die Zersetzung von *E. coli*- und *Staphylococcus*-Biofilmen zu konstruieren. Das in allen drei Strategien verwendete Anti-Biofilm-Agens ist Dispersin B. Dieses Enzym hydrolysiert Poly-N-Acetyl-Glucosamin, welches ein Bestandteil der Matrix der Zielbiofilme ist. Die Hydrolyse dieses Polymers führt zur Zersetzung des Biofilms.

In der ersten Strategie wird die N-Acetylglucosamin Signaltransduktion, sowie die chemotaktische Antwort in *E. coli* zu Hilfe genommen um den Zielbiofilm zu detektieren und Dispersin B zu synthetisieren. N-Acetylglucosamin induziert und erhöht hierbei die Synthese von Dispersin B. In der zweiten Strategie wird das Biofilm-spezifische Muster der Genexpression in *E. coli* ausgenutzt. Das gentechnisch veränderte Bakterium produziert Dispersin B durch die Aktivierung eines Promoters, der unter Biofilm Bedingungen mehrfach hochreguliert wird. Dabei wird dasBakterium selbst Teil Biofilms und zerstört diesen durch die Wirkung von Dispersin B. Die dritte Strategie zielt spezifisch auf dei Zerstörung des s. Epidermis Biofilms. Hierbei wird „Quorum Sensing“ in *S. epidermidis* ausgenutzt um den Zielbiofilm zu detektieren und um Dispersin B zu synthetisieren.

Die in dieser Arbeit vorgeschlagenen Strategien waren partiell erfolgreich. *E. coli* konnte gentechnisch verändert werden um Dispersion B zu exprimieren und zu sekretieren und um somit den Zielbiofilm effizient zu zerstören. Die vorgeschlagenen Strategien sind vielseitig anwendbar und können genetisch modifiziert werden um in ähnlichen biologischen Systemen gegen Biofilime andererSpezien wirksam zu werden. Im Rahmen dieser Arbeit habe ich unerwartete aspekte des N-Acetylglucosamin Signalweges in *E. coli* untersucht. Die Transkriptomanalyse von *E. coli* Biofilmen hat eine Hochregulierung von Redox-Stress assoziierten Genen in angehefteten Zellen ergeben. Dieser interessante Aspekt sollte weiterhin hinsichtlich der bakteriellen Signaltransduktion und der Biofilmbildung untersucht werden.

Abstract

Biofilms are aggregates of bacteria embedded in a self-produced matrix. In nature most bacteria exist in the form of biofilms. They are exceptionally resistant to environmental stress. They can survive harsh conditions such as starvation and desiccation, and can withstand conventional antimicrobial agents. Bacteria in the form of biofilms cause many diseases in humans, animals and plants. Formation of biofilms leads to corrosion of pipelines incurring huge losses in industries. The major focus of the research in microbiology is now shifting to biofilms. Researchers are exploring various strategies to combat biofilms.

Among different methods of combating biofilms, biological methods offer some advantages over other methods. The biological methods make use of the existing signaling pathways in bacteria and bring about efficient disruption of the target biofilm without having to add any artificially synthesized compound. These methods are particularly attractive in treating biofilm-induced diseases. In this project I have proposed three different strategies of engineering *Escherichia coli* to disrupt the biofilms of *E. coli* and *Staphylococcus epidermidis*. The anti-biofilm agent used in all the strategies is Dispersin B, an enzyme that hydrolyzes poly-N-acetyl glucosamine found in the matrix of the target biofilms. Hydrolysis of this polymer leads to disruption of the biofilm.

The first strategy makes use of the N-acetyl glucosamine signaling and chemotaxis pathway of *E. coli* to detect the target biofilm and to synthesize Dispersin B. The product of the action of Dispersin B is N-acetyl glucosamine, which acts as an inducer to elevate the synthesis of Dispersin B. The second strategy exploits the biofilm-specific pattern of gene expression in *E. coli* biofilms. The engineered bacterium expresses Dispersin B from the promoter of a gene that is multi-fold activated when the bacterium acquires the biofilm-lifestyle. It incorporates itself in the target biofilm and disrupts the biofilm through the action of Dispersin B. The third strategy is specifically aimed at disrupting *S. epidermidis* biofilms. It uses the *agr* quorum-sensing system of *S. epidermidis* to detect the target biofilm and to synthesize Dispersin B.

The strategies proposed in this project have been partially successful. I have demonstrated that *E. coli* can be engineered to express and secrete Dispersin B, which can disrupt the target biofilm efficiently. The strategies proposed in this project are versatile in application. They can be modified to engineer similar biological systems against biofilms of other species of bacteria. In the course of accomplishing the objective of this project I have explored some unknown aspects of the N-acetyl glucosamine signaling in *E. coli*. The analysis of the transcriptome of *E. coli* biofilms has revealed the up regulation of redox stress-associated genes in the attached cells of *E. coli* biofilms. These interesting findings can lead us to explore further into bacterial signaling and biofilm formation.

Contents

Part I.

Introduction

1. Biofilms – what we know so far

In the late 17th century Antonie van Leeuwenhoek having viewed human dental plaque under a self-made microscope, recorded observations that would be regarded as a pioneering for modern Microbiology. In one of his letters to the Royal Society of London he mentioned "*the number of these animalcules in a scurf of man's teeth are so many that I believe they exceed the number of men in a kingdom*". Since then microbiology has grown vast. However, the field focused for the majority of its history on pure cultures of bacteria suspended in shaken liquid. In the mid 20th century, some researchers realized that most bacteria in nature survive in the form of slime or as films attached to surfaces (Heukelekian & Heller, 1940). Such aggregates of bacteria attached to a surface were later defined as biofilms. What Leeuwenhoek had seen in a dental plaque was actually the very first microscopic observation of a biofilm. By the late 20th century, scientists had developed tools and techniques to study these bacterial aggregates, and the study of biofilms has since evolved into a central focus of microbiology.

Biofilms are aggregates of microbial cells embedded in a self-produced matrix. They grow on a wide variety of biotic and abiotic surfaces such as living tissues, indwelling medical devices, inner surfaces of pipes, surfaces of submerged rocks. They are exceptionally tolerant to environmental stresses including desiccation, lack of nutrients, shear-forces and attack by hosts' immune system. With the advent of Confocal Laser Scanning Microscopy (CLSM) it has been possible to study the structure of biofilms in detail. Biofilms are now known to possess complex three-dimensional structures that are critical to many of their collective properties. Figure 1.1 provides a brief summary of a typical biofilm life cycle (Stoodley *et al.* , 2002). Planktonic cells attach to a surface irreversibly, begin secreting extracellular matrix and grow to produce a micro-colony. These micro-colonies grow further and form a complex three-dimensional structure. Within a mature biofilm, cells differentiate to perform different functions. Spontaneously or in response to stresses such as starvation, some cells detach from biofilms back into the planktonic phase, allowing them to disperse and form a biofilm somewhere else. A great deal of information is now known regarding molecular mechanisms of biofilm formation and maturation (López *et al.* , 2010; Karatan & Watnick, 2009).

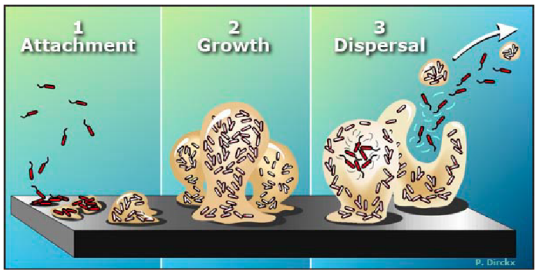

Figure 1.1. – Schematic of biofilm formation (The Image library of Center for Biofilm Engineering, Montana State University)

1.1. Why do bacteria form biofilms?

The growth rate of bacteria that are embedded in a matrix is certainly lower than that of planktonic cells. However, in nature most bacteria are seen to exist in a biofilm lifestyle. The biofilm lifestyle offers several other advantages to bacteria that counter-balance the disadvantage of slowed growth. Attachment to a surface makes bacteria stay in their preferred niche. For example, oral *Streptococci* usually tend to bind to dental pellicle and make biofilms. If they existed in planktonic state, saliva would easily wash them away (Kreth *et al.* , 2009). Reduced rate of growth also allows bacteria to survive conditions of nutrient limitation. A biofilm offers an excellent environment for horizontal gene transfer (Madsen *et al.* , 2012). Within a biofilm cells can differentiate and show a division of labor (Kolter *et al.* , 2015). In some cases different species of biofilms form a mixed biofilm and perform metabolic functions that are cooperative. The best example of such symbiosis is the colonization of the oral cavity by *Streptococci*. During initial stages of colonization, aerobic or facultatively anaerobic bacteria attach first. This initial colonization creates an anaerobic microenvironment that provides a niche for the attachment of the anaerobic bacteria (Ruby & Goldner, 2007). Scientists have considered a biofilm as an analogue of a multicellular organism (Nikolaev & Plakunov, 2007).
Considering all these factors it is clear that the biofilm lifestyle provides bacteria with numerous survival advantages in their natural habitats. Although most biofilms in nature pose no threat to human life, some can be notoriously dangerous to human health and also cause a huge economic loss as agents of industrial fouling.

1.2. Biofilms in industries

Bacteria tend to form biofilms on any surface with sufficient moisture and supply of organic material. Microbial biofilms clog water filtration units affecting drinking water supply, wastewater transport, desalinization units and industrial water-cooling systems. Biofilms corrode and block pipelines and interfere with oil and gas extraction processes.

Different mechanisms of biofilm-induced corrosion have been reviewed extensively (Videla, 2001). In the marine shipping industry, biofilms that form on ship hulls lead to corrosion and cause increased drag, which results into higher consumption of fuel during transport as well as higher ship hull maintenance costs (Dobretsov *et al.* , 2013). A wide number of pathogenic bacteria form biofilms in food processing industries. Certain pathogenic strains of *Escherichia coli* are known to be present on apple surfaces, which compromise the safety of raw materials used in juice processing industries (Cody *et al.* , 1999). In total, industrial biofouling and bio-corrosion cause over $200 billion in damage per year in the USA alone. Bacterial diseases associated with biofilm formation also devastate agricultural crops. For example, the Gram-negative bacterium *Erwinia amylovora* causes a biofilm-induced disease called fireblight on Apple and Pear plants, resulting in multimillion Euro damage every season (Vrancken *et al.* , 2013).

1.3. Biofilms in diseases

Bacteria form biofilms in human and animal hosts and cause a wide range of diseases that are difficult to treat.

1.3.1. *Pseudomonas aeruginosa* biofilms in cystic fibrosis patients

The most studied example is that of cystic fibrosis (CF), an autosomal recessive genetic disorder that affects the lungs, pancreas, liver and intestines. It is caused by a mutation in the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, which results into dysfunctional release of mucus. Accumulation of mucus in different organs, especially in the lungs, provides hostile environment for biofilm formation by *Pseudomonas aeruginosa*. These biofilms are resistant to conventional antibiotics and the host immune system, and lead to chronic infection in the majority of CF patients (Davies, 2002). Because of the severity of the infection, *P. aeruginosa* biofilm has been a major focus of the research and a great deal is known about it (Tolker-Nielsen, 2014).
P. aeruginosa is a Gram-negative, rod shaped bacterium found widely in the environment. It rarely causes infections in healthy individuals; however it can multiply freely in immune-compromised people. *P. aeruginosa* has been a model organism to study biofilm formation. The matrix of *P. aeruginosa* biofilms contains two major classes of polysaccharides- capsular and aggregative. Capsular polysaccharides decorate the exterior of the cells whereas aggregative polysaccharides interact with other components of the matrix and impart structural integrity to the biofilm (Mann & Wozniak, 2012). Alginate is a capsular polysaccharide, found in mucoid strains of *P. aeruginosa*, which is composed of β, 1⇒4 linked residues of L-guluronic acid and D-mannuronic acid (Evans & Linker, 1973). Alginate has

been implicated in persistence and immune evasion (Leid *et al.* , 2005). Pel and Psl are aggregative polysaccharides. Psl is a heterogeneous polymer of D-mannose, D-glucose and L-rhamnose. Overexpression of Psl causes excessive aggregation of cells indicating that Psl is a main component of the matrix contributing to the structural integrity. Pel is a polysaccharide of undefined structure. Pel also contributes to the structural integrity of the biofilm; however, its role is secondary to that of Psl (Colvin *et al.* , 2012). Apart from these polysaccharides, the biofilm matrix of *P. aeruginosa* also contains extracellular DNA that makes a significant contribution to its structural stability (Allesen-Holm *et al.* , 2006). The exceptional resistance of *P. aeruginosa* biofilms is attributed to immune-evasive properties of its matrix components and extremely slow-growing state of cells in the infected CF patient.

P. aeruginosa biofilms have a three dimensional structure consisting of microcolonies formed in a mushroom-like shape, separated by fluid-filled channels. Cells within the biofilm communicate with each other through signaling molecules called acyl homoserine lactones. Presence of this signaling mechanism is essential for maintaining the three-dimensional structure of the biofilm (Davies *et al.* , 1998).

1.3.2. *Staphylococcus epidermidis* biofilms in device-related infections

Another example of medically relevant biofilms is device-related infection. Intravenous catheters, peritoneal dialysis catheters, prosthetic heart valves, joint prostheses, cerebrospinal fluid shunts save lives of millions, but all have intrinsic risk of surface-associated infections. These infections are highly resistant and difficult to treat. While they do not lead to any life-threatening disease, the cost of the treatment puts a severe burden on public health system. The costs related to such treatment amount to $2 million per year in the USA alone (Otto, 2008).

Staphylococcus epidermidis is a Gram-positive, coccoid bacterium that is commonly found on the human skin. It does not cause any infections in healthy individuals (Kloos & Musselwhite, 1975). However, when it gains access to medical implants it causes severe infections (Von Eiff *et al.* , 2002). It is one the highly significant pathogen infecting medical implants. Biofilm formation by *S. epidermidis* has been studied in detail and extensive reviews are available (O'gara & Humphreys, 2001; Otto, 2008).

In comparison to *S. aureus*, virulence factors found in *S. epidermidis* are few and biofilm formation is the most important mechanism associated with infection. *Staphylococci* produce unique adhesive proteins called MSCRAMMs (Microbial Surface Components Recognizing Matrix Molecules) that bind to human matrix proteins such as fibrinogen and fibronectin. SdrG from *S. epidermidis* is the most studied MSCRAMM. It has been shown to bind to fibrinogen and it is involved in intercellular adhesion and aggregation. Apart

from SdrG, other proteins like SdrF, Aap and EmbP are also known to have similar roles. Another important component of *S. epidermidis* biofilm matrix is polysaccharide intercellular adhesin (PIA). This is a polymer of β, 1⇒6 linked N-acetyl glucosamine. It is alternatively referred to as PNAG. The enzymes encoded from the *icaABCD* operon synthesize PNAG. It is mainly involved in immune evasion and biofilm accumulation. It also contributes to the architecture of the biofilm (Rohde *et al.* , 2010). Deletion mutants of *icaABCD* genes are unable to form 'towers' and 'mushrooms' in the biofilm. In spite of being a significant component of the matrix, in case of some clinical isolates of *S. epidermidis*, PIA is not synthesized. The biofilm formation in those isolates is more dependent on the adhesive protein Aap. Like *P. aeruginosa*, *Staphylococci* use quorum-sensing controlled surfactant peptides called phenol-soluble modulins (PSMs) to maintain the structure of the biofilm (Yarwood *et al.* , 2004).
Researchers have reviewed various diseases caused by bacterial biofilms (Costerton *et al.* , 1987). Diseases involving biofilms often become chronic and difficult to treat. Researchers are now studying the exact nature of biofilm infections in order to find ways to treat them (Bjarnsholt *et al.* , 2013).

1.4. What makes biofilms stress-resistant?

As noted above, residing in a biofilm confers resistance to many of the stresses incurred in harsh environments. Being attached and embedded in a slimy material protects the cells from desiccation and starvation. There are two main hypotheses regarding biofilms' exceptional resistance to environmental stress. The first is that the extracellular matrix acts as a barrier and prevents penetration of antimicrobial agents. The polymeric nature of the matrix alters diffusion of small molecules within a biofilm (Stewart & William Costerton, 2001). Antimicrobial agents such as superoxides, antibiotics, detergents, surfactants and immunoglobulins are either blocked, neutralized or diluted to sublethal concentrations before they reach individual bacterial cells within a biofilm. The second mechanism suggests that at least some cells in a biofilm exist in a dormant, slow-growing state. While some antibiotics can penetrate the biofilm matrix, they are ineffective against these dormant cells. Antibiotics require at least some degree of metabolic activity to be effective since they target such activities to kill the cells. Formation of dormant cells is one of the significant factors contributing to exceptional resistance of biofilms (Brown *et al.* , 1990).

1.5. Differential expression of genes in biofilms

The biofilm-lifestyle of bacteria is fundamentally different than the planktonic lifestyle. The drastic phenotypic changes observed in biofilms led to the hypothesis that physiolog-

ical modifications involved in transitioning to the biofilm mode of life must be regulated through changes in gene expression.

The earliest evidence supporting this hypothesis came from gene-fusion studies, which suggested that the expression of approximately 38% of bacterial genome might be affected during biofilm formation (Prigent-Combaret *et al.* , 1999). However, studies based on DNA-microarray methods indicated that only 5 to 10% of the genome might be expressed differently during biofilm formation (Schembri *et al.* , 2003; Ren *et al.* , 2004). So far such studies have been performed with *E. coli, P. aeruginosa, S. aureus, Bacillus subtlis* and some other bacteria. Researchers have not found any specific pattern of gene expression that is common to all biofilms.

However, certain trends in gene expression in biofilms observed in different species have been reviewed (Beloin & Ghigo, 2005). The switch from planktonic to sessile growth phases requires down-regulation of genes related to motility and up-regulation of genes encoding adhesive polymers. The metabolic state of cells within a biofilm is similar to that of cells in stationary phase. Cells in a biofilm show induction of genes related to stress-response. Every biofilm is perhaps a world of its own, implying a unique pattern of gene expression. What is true for *E. coli* biofilms may not be true for *P. aeruginosa* biofilms. The pattern of gene expression within a biofilm is variable, both spatially and temporally. Studying expression of genes in various micro environments within a biofilm at different intervals of time can be more informative rather than transcriptomic analysis of the whole biofilm biomass.

2. *Escherichia coli* biofilms

E. coli is the most widely studied bacterium. It is a Gram-negative, rod-shaped, facultative anaerobe that inhabits in the gut of humans and other mammals. Although it is a harmless commensal, some strains of *E. coli* have acquired certain virulence attributes, which confer an ability to colonize other niches and cause a wide spectrum of diseases. Three main categories of infections caused by pathogenic *E. coli* are enteric/diarrheal diseases, urinary tract infection and sepsis/meningitis (Kaper *et al.* , 2004). Many isolates of *E. coli* are able to form biofilms in vivo and in vitro. Due to ease of handling, it has been extensively used for understanding biofilms. Different events from surface attachment to formation of a mature biofilm have been reviewed in detail (Beloin *et al.* , 2008). Since the extracellular matrix is the most important part of a biofilm, it is described here more in detail.

2.1. *E. coli* biofilm matrix

The *E. coli* biofilm matrix predominantly contains exopolysaccharides- cellulose, PGA and colanic acid. The matrix also contains cellular appendages like flagella, type 1 fimbriae and proteinaceous curli fibers. These components make the structural scaffold of the biofilm. It also contains limited amounts of lipopolysaccharides and capsules to a small extent, which may have an indirect role in biofilm formation. Figure 2.1 shows a schematic representation of the biofilm matrix of *E. coli*.

PGA (poly-n-acetyl glucosamine)

PGA is a homopolymer of N-acetyl glucosamine residues connected by β, 1⇒ 6 linkages. Around 10% of the residues are de-acetylated. This polymer is the same as PNAG found in Staphylococci. Enzymes encoded by the operon *pgaABCD* synthesize this polymer. Among the genes present on the operon, *pgaC* and *pgaD* encode inner membrane proteins responsible for synthesis of the polymer, whereas *pgaA* and *pgaB* encode outer membrane proteins involved in export of the polymer. PGA is primarily cell-associated and localized at cell poles (Itoh *et al.* , 2008). The deletion mutant of PGA forms less biofilms (Niba *et al.* , 2007). Lack of PGA does not completely abolish the ability of the cells to attach to a surface. However, it affects the process of transition from temporary to permanent attachment (Agladze *et al.* , 2005).

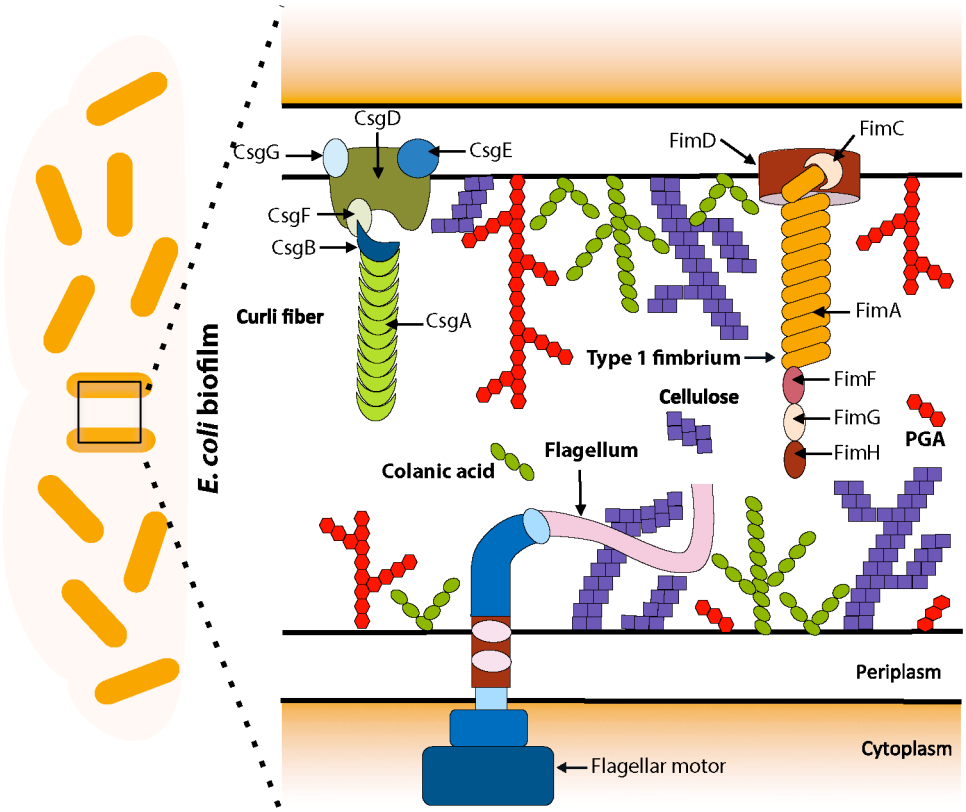

Figure 2.1. – The biofilm matrix of *E. coli*. (Figure adapted from Kostakioti *et al.* (2013))

Cellulose

Cellulose is a homopolysaccharide composed of D-glucopyranose units linked by β-1⇒4 glycosidic bonds. It is expressed from the *bcsABZC* operon. *E. coli* harbors the genes responsible for cellulose synthesis, however, the most common laboratory strain *E. coli* K12 is not known to produce cellulose. Certain wild type isolates of *E. coli* have been shown to produce cellulose under specific conditions. Co-expression of cellulose with thin aggregative fimbriae dramatically alters the morphology of the biofilm (Zogaj *et al.* , 2001). In short, cellulose is more relevant in three-dimensional structure of the biofilm than initial attachment of the cells.

Colanic acid

Colanic acid is a negatively charged polymer of glucose, galactose, fucose and glucuronic acid that forms a protective layer on the bacterial cell under specific conditions. Its synthesis involves 19 genes located in the same cluster named *wca*. Colanic acid does not enhance adhesion of bacterial cells to the surface but rather blocks the cell-surface interactions. However, expression of colanic acid is up regulated in mature biofilms, indicating that its synthesis is involved in shaping the structure of the biofilm (Hanna *et al.* , 2003).

Type 1 Fimbriae

Type 1 fimbriae (or pili) are filamentous proteinaceous adhesins commonly expressed by several strains of *E. coli* (Sauer *et al.* , 2000). There are normally 100 to 500 type 1 fimbriae present on a bacterial cell surface. Fimbriae are tubular structures, around 5 to 7 nm in diameter and 0.2 to 2 µm long. They can adhere to various receptor molecules on eukaryotic cell surface and also to some abiotic surfaces. They are synthesized from the *fimBEAICDFGH* operon. Within this operon *fimB* and *fimE* encode regulatory proteins; *fimA, fimG, fimF* and *fimH* encode structural proteins of fimbriae and others make proteins involved in their synthesis. A typical fimbria is a right-handed helical rod composed of around 1000 FimA residues with a tip made up of the adapter proteins FimG and FimF and the adhesin FimH (Schilling *et al.* , 2001). A deletion mutant of *fimA* is severely compromised for attachment to surfaces and thus is defective in biofilm formation (Beloin *et al.* , 2004).

Curli fibers

Curli fibers are similar to fimbriae in structure and function. They are proteinaceous tubular structures present on the *E. coli* cell surface. They are 0.5 to 1 µm long and 6 to 12 nm in diameter. Curli fibers attach to proteins of the extracellular matrix of human tissues such as fibronectin, laminin and plasminogen and thus promote attachment of bacterial cells to different human cells (Nasr *et al.* , 1996). They also promote biofilm formation on abiotic surfaces (Uhlich *et al.* , 2006). Genes involved in curli synthesis are clustered in the *csgBA* operon, which encodes curli structural components, and the *csgDEFG* operon, which encodes a transcriptional regulator (CsgD) and curli export machinery (CsgE-G). Expression of curli is cryptic in the most common laboratory strains of *E. coli* whereas in other isolates it is under tight transcriptional regulation (Brombacher *et al.* , 2003).

Flagella

A flagellum is a lash-like extracellular appendage of *E. coli* that functions as a locomotive organ. An *E. coli* cell has around 6 flagella arranged peritrichously over its surface. A typical flagellum is 15 to 18 nm in diameter and 5 to 10 µm long. Synthesis of a flagellum involves around 50 genes and is regulated by various factors. Flagella are involved in attachment of cells to a surface (Giron *et al.* , 2002), and cells lacking flagella are deficient in attachment and subsequent biofilm formation (Niba *et al.* , 2007).

Cell surface polysaccharides

Besides released polysaccharides like PGA and colanic acid, cell surface-associated polysaccharides have also been identified to play roles in biofilm formation. Lipopolysaccharide (LPS) is a glycolipid polymer that constitutes the main component of the outer membrane of *E. coli* cell. It is synthesized by the action of more than 50 genes. Mutations affecting LPS synthesis often affect the ability of *E. coli* cells to adhere to surfaces (Genevaux *et al.* , 1999). However, since defects in LPS synthesis affect type 1 fimbriae expression and motility, reduced biofilm formation by LPS synthesis mutants could be due to indirect effects. *E. coli* cells also possess surface-enveloping structures composed of high molecular weight capsular polysaccharides that are firmly attached to the cell. They are proven to be virulence factors and protect the cells from attack by immune cells. Some capsular polysaccharides are released into the medium and display anti-adhesion activities towards both Gram-positive and Gram-negative bacteria. These polysaccharides weaken the interactions between cells and surfaces and drastically reduce biofilm formation. They can be used to prevent attachment of nosocomial pathogens (Valle *et al.* , 2006).

2.2. Biofilms of different WT strains of *E. coli*

Several strains of *E. coli*, that are designated as WT strains are in use in the scientific community. The strain K-12 was isolated from a diphtheria patient in Palo Alto, California in the year 1922. This strain was deposited in Stanford University and given the name- 'K-12' (Bachmann, 1996). Several other sub-strains were derived from this strain. The strains MG1655 and W3110, which are derivatives of K-12 are closely related. Recent whole-genome sequencing of these two strains has revealed that W3110 carries mutations in eight sights in comparison to MG1655 (Hayashi *et al.* , 2006). Some lineages of W3110 acquired a mutation in the CDS of *rpoS*, which resulted in the non-functional Sigma S transcription factor (Jishage & Ishihama, 1997). Sigma S is a subunit of RNA polymerase, which transcribes the genes involved in general stress response and secondary metabolism (Maciag *et al.* , 2011). This strain, with non-functional Sigma S, is referred to as W3110 in this thesis.

A certain lineage of W3110 is known for having functional Sigma S. This strain is regarded as W3110RH in this thesis. Derivatives of *E. coli* K12 do not produce cellulose in spite of containing genes encoding enzymes required for cellulose biosynthesis (Zogaj *et al.* , 2001). Researchers figured out that a single mutation in the bcs operon had resulted in the lack of production of cellulose. They 'cured' this mutation and derived a strain of *E. coli* that synthesizes cellulose (Serra *et al.* , 2013). This strain is regarded as W3110AR in this thesis.

2.3. The *crsA* mutant of *E. coli*

There are several regulatory processes involved in biofilm formation. CsrA (Carbon Storage Regulation), for example, regulates several unrelated pathways, including central carbon metabolism, motility, biofilm formation, virulence, pathogenesis, quorum sensing, and oxidative stress response etc. (Timmermans & Van Melderen, 2010). It is a 61 amino acid protein that binds to mRNA and regulates its stability and translation. It acts mostly negatively by inhibiting the translation of mRNA and thus regulates the pathway post-transcriptionally (Romeo *et al.* , 1993). There are two sRNAs called CsrB and CsrC, which bind and sequester CsrA, thereby inhibiting its activity (Weilbacher *et al.* , 2003).

The functions of CsrA are essential for survival of the cell. It is not possible to make a deletion mutant of *csrA*. However, scientists were able to generate a transposon-insertion mutant of *csrA*, which was not only viable but also formed prolific biofilms. The growth rate of this mutant was the same as that of the wild type, and biofilms made by this mutant were robust, but normal in appearance. Overexpression of CsrA led to the opposite result, namely decreased biofilm formation (Jackson *et al.* , 2002). The effect on biofilm formation is due to de-repressed synthesis of PGA, a component of the extracellular matrix of *E. coli*. CsrA binds to *pgaABCD* transcript, which is responsible for synthesis of PGA. Binding of CsrA lowers the translation rate of the *pgaABCD* transcript resulting in repression of PGA synthesis (Wang *et al.* , 2005). The inhibition of translation of *pgaABCD* by CsrA is counteracted by McaS. It also binds to the 5' untranslated region of the *pgaABCD* transcript, however, it opens up the secondary structure of mRNA and allows better binding of the ribosome (Jorgensen *et al.* , 2013).

CsrA also represses the translation of YcdT and YdeH, which are GGDEF-containing proteins mainly responsible for the synthesis of cyclic-di-GMP (Jonas). Many processes in the cell that lead to biofilm formation require the second messenger cyclic-di-GMP. In general, more cyclic-di-GMP implies more likelihood of biofilm formation and vice versa (Jonas *et al.* , 2008). By inhibiting the synthesis of cyclic-di-GMP, CsrA reduces the likelihood of biofilm formation. CsrA is also known to stabilize the mRNA of *flhDC*, the master regulator of flagellum biosynthesis, thereby making the cells more motile (Yakhnin *et al.* , 2013). From these different findings, it is clear that CsrA regulates the switch from planktonic to sessile lifestyle. It drives the cell more towards motility and reduces its chances of acquiring the sessile lifestyle.

The non-lethal mutant version of *csrA* (*E. coli* TRMG1655) is defective in its binding action. Thus, the *csrA* mutant strain of *E. coli* exhibits elevated synthesis of PGA and increased biofilm formation (Romeo *et al.* , 1993). This mutation mimics the state of the cell in which the cell is switching to the biofilm lifestyle.

2.4. Differential regulation of gene expression in *E. coli* biofilms

The physiological state of a biofilm is thought to be an outcome of a coordinated pattern of gene expression. Various studies performed on different species of bacteria have supported this hypothesis (section 1.5).

So far, 8 such studies have been performed for *E. coli* biofilms. Schembri and coworkers studied differential gene expression within exponential phase cultures, stationary phase cultures, and one-day old biofilms of *E. coli* grown in flow-chambers. They used DNA microarray-based technique to quantify the levels of different mRNA molecules present in the cells. They found that a very small percentage of genes is significantly altered in biofilms in comparison to other planktonic cultures of either growth phases. They observed that genes encoding proteins involved in adhesion, such as type 1 fimbriae and antigen 43 are highly expressed in biofilms. Along with adhesive factors, genes encoding (putative) transport proteins, putative oxidoreductases and genes associated with heavy metal resistance were also induced in biofilms. Unfortunately most of the genes that were significantly altered were not identified and not assigned any definite function (Schembri *et al.* , 2003).

Beloin and coworkers studied gene expression in 8-day old mature biofilms grown in a bioreactor. They observed that the pattern of gene expression in a biofilm is very much similar to that of a stationary phase culture. Their general observations are summarized in figure 2.2. They provided evidence that the expression of genes associated with envelop stress response (*psp* operon, *rpoE* and *cpx* pathways) is a general feature of *E. coli* mature biofilms. They performed functional profiling of the identified genes by creating deletion mutants and observing their effects on biofilm formation. They also constructed transcriptional reporters of these genes and studied their spatial and temporal pattern of expression (Beloin *et al.* , 2004).

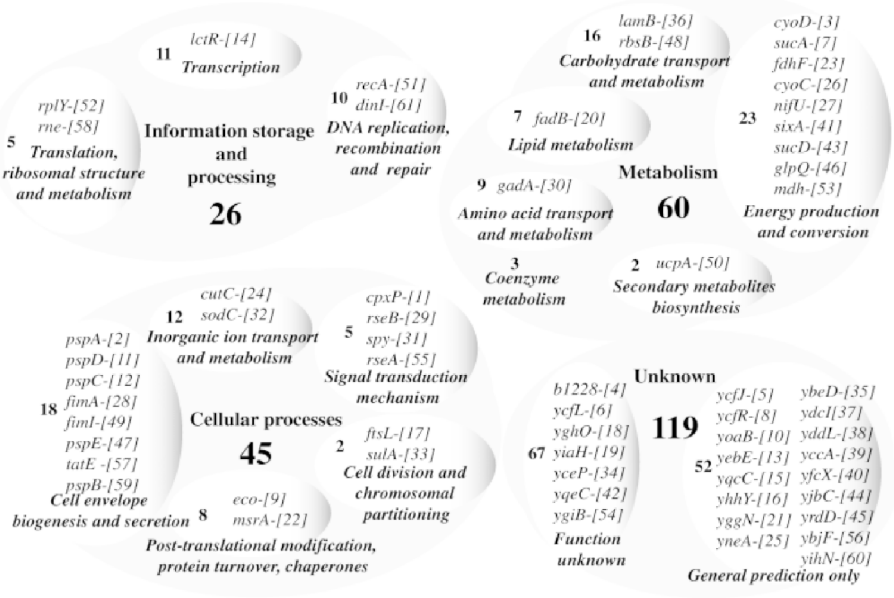

Figure 2.2. – Genes up regulated in biofilms (Beloin *et al.* , 2004)

Ren and coworkers also performed a similar study on biofilm grown for different intervals of time. Although they confirmed the induction of type 1 fimbriae, antigen 43 and genes associated with stress response, they found many other genes significantly altered which were not in agreement with findings of Schembri and coworkers. They concluded that the pattern of gene expression depends on the strain, media and conditions of biofilm formation (Ren *et al.* , 2004).

Researchers have also realized that the physiological state of cells within a biofilm is highly heterogeneous. This could be due to differences in availability of oxygen and nutrients in all parts of a biofilm. These differences are also reflected in the pattern of gene expression. When cells from a biofilm are harvested for mRNA isolation, this spatial heterogeneity is not taken into account. This results in poorer understanding of the biofilm-associated pattern of gene expression.

Besides studying the pattern of gene expression, it is also interesting to look at the genes essential for biofilm formation. Niba and colleagues screened a collection of single-gene deletion mutants of *E. coli* to identify the genes directly involved in biofilm formation (Niba *et al.* , 2007). Out of 3985 deletion mutants of non-essential genes that were examined in this study, 110 were found to result in reduced biofilm formation. The majority of these 110 genes were those involved in flagellar biosynthesis, biogenesis of adhesive structures like type 1 fimbriae, curli and lipopolysaccharides. As stated by the authors, there could be other genes involved in biofilm formation, which are required in some other conditions. Since gene-expression within biofilms is heavily dependent on conditions of growth, further investigation is required to identify such genes.

3. Methods of combating biofilms

Due to huge economic losses caused by biofilms and their considerable stress resistance, combating biofilms is a major outstanding challenge for researchers. As new aspects about biofilm formation are discovered, novel strategies of combating them are continually being explored. The following is a brief review of various strategies that researchers have explored to combat biofilms. These strategies are broadly classified into physicochemical methods, biochemical methods and biological methods.

3.1. Physicochemical methods

Physicochemical methods involve physical or chemical destruction of biofilms. These methods are more relevant in industrial settings. The most common methods are treatment with hot water, detergents, common disinfectants or sonication. Another approach is to modify surfaces in order prevent attachment of bacteria. For example, electro-polished stainless steel reduced the attachment of bacteria and subsequent biofilm formation (Arnold & Bailey, 2000). Coating surfaces with immobilized lysozyme also significantly prevented the attachment of bacteria (Yuan *et al.* , 2011). Use of detergents was also employed in a healthcare setting. Researchers evaluated different detergents for their efficiency to remove biofilms from endoscopes (Vickery *et al.* , 2004).

3.2. Biochemical methods

Biochemical methods are those in which a biologically derived compound is used to eradicate biofilms. The mechanism of action of this agent can be specific to a particular biofilm or a particular stage of biofilm development. Bacteria use quorum sensing to coordinate communal behaviors such as bioluminescence, virulence and in some cases biofilm formation (Miller & Bassler, 2001). The most commonly used compounds in quorum sensing are acyl homoserine lactones (AHLs). Researchers have identified enzymes, which hydrolyze AHLs and disrupt bacterial communication (Chen *et al.* , 2013). Such enzymes have been used to coat surfaces and prevent biofilm formation (Kim *et al.* , 2014).
As described above, extracellular matrix is probably the major hindrance in the action of antimicrobial agents. Researchers have shown that lipopeptide biosurfactant produced by Bacillus licheniformis significantly enhances the efficacy of antibiotics used in killing *E. coli*

biofilms (Rivardo *et al.* , 2009). Micelle-encapsulated antibiotics are reported to kill biofilm cells (Cheow & Hadinoto, 2014). Some biofilm forming bacteria also secrete enzymes that can degrade a certain component of the matrix. Such enzymes are necessary for the turnover of organic material within a biofilm. Researchers have combined conventional antimicrobial agents with such enzymes to enhance their penetration into the biofilm. Aminoglycosides administered along with DNAseI and alginate lyase helped is reducing *P. aeruginosa* cells in CF patients (Alipour *et al.* , 2009).

3.3. Biological methods

Biological methods make use of bacteria or phages to attack a biofilm. In nature, several species of bacteria co-exist. In order to outcompete each other, bacteria produce certain compounds, which are detrimental to other species. For example, some strains of *E. coli* produce colicins, which lyse the cells of other species, however protect the cells of their own (Cascales *et al.* , 2007). The early dental plaque colonizer *Streptococcus gordonii* secretes proteases that reduce subsequent colonization of *S. mutans* (Wang *et al.* , 2011). Bacteriophages are natural enemies of bacteria. They can also be used to target a biofilm. The real potential of these abilities of bacteria can be exploited by engineering them in order to target a specific biofilm and eradicate the biofilm effectively.

Researchers have engineered phages to express a biofilm-degrading enzyme that effectively kills the biofilm (Lu & Collins, 2007). Phages expressing quorum-quenching enzymes have been reported to lyse bacterial cells in a biofilm and inhibit the signaling of other bacteria in the vicinity (Pei & Lamas-Samanamud, 2014). Researchers have designed a 'pathogen-sense and kill' system in which engineered bacteria sense the presence of a target biofilm through quorum sensing system and secrete an anti-biofilm compound that eventually destroys the target biofilm (Hwang *et al.* , 2013).

The choice of the method depends on the kind of organism and location of a biofilm. Physicochemical methods are more relevant to industrial settings whereas biochemical methods are relevant in treating diseases. These methods do have some advantages in their respective applications; however, none is proven to be the most efficient and cost effective method. Biological methods in which phages or bacteria are engineered to disrupt biofilms are comparatively new and still under trials for actual use. However, they do offer certain advantages over physical and chemical methods. These methods mainly exploit a signal transduction pathways underlying biofilm formation or dispersal. They can function without adding an external inducing agent. Their action can be specific as well as versatile at the same time. Biological methods can be combined with biochemical or physicochemical methods to have better results. With existing strategies having various limitations, biological methods are offering a novel and efficient way to combat biofilms.

3.4. Matrix-degrading enzymes

Many bacteria are known to possess secreted enzymes in their biofilm matrix. These enzymes degrade polymeric substances in the biofilm matrix. They are involved in recycling of matrix components and thus maintain the dynamic nature of the biofilm (Kaplan, 2014). Since they are enzymes produced by bacteria themselves, they are non-toxic, synthesized in a simple manner, can be expressed in a bacterial host and readily secreted out of the cells. These properties of matrix-degrading enzymes make them compatible with biological methods of biofilm disruption. Researchers have identified therapeutic potential of matrix-disrupting enzymes (Kaplan, 2009) and also successfully shown that they can be efficient in disrupting biofilms under experimental setting.

The extracellular matrix constitutes 90% of the biomass of a biofilm and provides the structural scaffold on which the biofilm forms. The matrix also retains water and nutrients and prevents desiccation of the biofilm. It acts as a barrier to antimicrobial agents. Thus, it is the most essential aspect of biofilms' existence. Matrix-degrading enzymes are mostly peptidases, glycosidases or deoxyribonucleases, which respectively target proteins, carbohydrates, and DNA present in the biofilm matrix. Degradation of components of the matrix leads to loss of the structural scaffold and eventual dispersal of the biofilm. There is no universal enzyme, which can disperse all kinds of biofilms. However, if a certain enzyme is able to degrade the most important component of a biofilm, it is likely to successfully disperse that biofilm. For example, alginate lyase is able to disperse *P. aeruginosa* biofilm by degrading alginate in its matrix (Boyd & Chakrabarty, 1994). SPRE (surface-protein-releasing enzyme) can disperse the biofilm of *S. mutans* by degrading salivary receptor P1, which is responsible for attachment of cells to the tooth surface (Lee *et al.* , 1996). Dispersin B is able to disperse biofilms of wide range of bacteria by degrading poly-N-acetyl glucosamine (PGA) in their matrix (Itoh *et al.* , 2005). Researchers have identified several such enzymes, which are either specific to a particular biofilm or effective against a wide range of biofilms. Due to their ease of expression in a bacterial host and effectiveness against biofilms, matrix-degrading enzymes are suitable anti-biofilm candidates.

3.5. Dispersin B

Among various matrix-degrading enzymes discovered so far, Dispersin B has drawn a perhaps the most attention. Dispersin B is an enzyme isolated from *Actinobacter actinomycetemcomitans*. This bacterium is Gram-negative, non-motile coccobacillus that colonizes the human oral cavity. It causes localized juvenile periodontitis, a severe form of periodontal disease that affects adolescents. Biofilms of this bacterium show a ubiquitous phenomenon of dispersal and formation of new biofilms in other locations. The cells secrete the enzyme, Dispersin B, which degrades the extracellular matrix and helps in the

dispersal of the biofilm (Kaplan *et al.* , 2003).

Dispersin B is a hexosaminidase and belongs to a family of glycosyl hydrolases. It hydrolyzes the β, 1⇒6 linkage of poly-N acetyl glucosamine (PGA) of bacterial extracellular matrix (Itoh *et al.* , 2005). The 40 kDa enzyme is 361 amino acids long and has a single domain which can be divided into major substructures. The major substructure is a TIM-barrel that can be further divided into minor substructures (Ramasubbu *et al.* , 2005). The active site is a large cavity in the center of bowl-shaped enzyme and contains Asp184, Glu183 and Glu322 as most conserved residues. The mechanism of action is similar to that of other glycosyl hydrolases. It cleaves terminal monosaccharide residues from the non-reducing end of the polymer (Manuel *et al.* , 2007).

The activity of the enzyme can be measured using chromogenic substrates (Chibba *et al.* , 2011). The most widely used substrate is 4-Nitrophenyl N-acetyl-β-D-glucosaminide (NP-GlcNAc) (Shibata & Yagi, 1996). This substrate mimics the β, 1⇒6 linkage found in PGA. Upon action of the enzyme 4-nitrophenoxide is released as a product, which can be quantified by reading absorbance at 400 nm. Figure 3.1 shows the structures of Dispersin B, NP-GlcNAc and PGA.

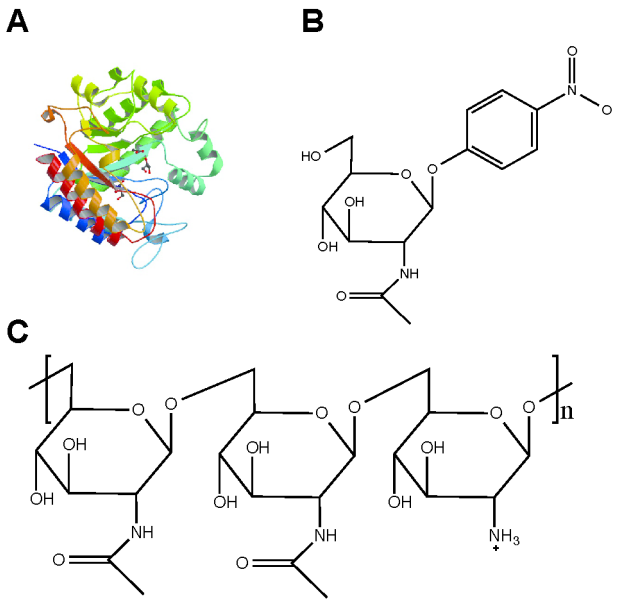

Figure 3.1. – **A**. Structure of Dispersin B (PDB ID 1YHT, Ramasubbu *et al.* (2005)), **B**. Structure of NP-GlcNAc, **C**. Structure of PGA

PGA is found in many species of Gram-positive and Gram-negative bacteria such as *E. coli*, *S. epidermidis*, *S. aureus*, *Yersinia pestis, P. fluorscense, Bordetella spp., Xanthomonas spp.,* and others. The biofilm-disrupting action of Dispersin B has been demonstrated on some of these species. Thus, it is possible to have widespread applications of Dispersin B as a biofilm-disrupting agent.

4. Signaling pathways in bacteria

Biological methods of combating biofilms involve engineering existing signal transduction pathways in such a way that the anti-biofilm agent is synthesized in response to a biofilm. Following is a description of some signaling pathways related to the components of *E. coli* biofilm matrix.

4.1. N-acetyl glucosamine and its metabolism in *E. coli*

N-acetyl glucosamine is a constituent of PGA, which is an important component of the biofilm matrix of *E. coli*. It is also found in various other Gram-positive and Gram-negative bacteria.

N-acetyl glucosamine is a derivative of glucose. It has an acetylamine residue attached to C-2 of its glucopyranose ring. It can make different forms of glycosidic linkages with other sugar molecules and become a part of a polymer. It is an important constituent of bacterial cell wall, which is made up of peptidoglycan. NAG makes β, 1⇒4 glycosidic bond with N-acetyl muramic acid to make the tough and insoluble backbone of peptidoglycan. As stated above, NAG is also a part of chitin, the most abundant polysaccharide in the marine habitat. Chitin is found in fungal cell walls, crustaceans' exoskeleton and mollusk shells. It is a polymer of NAG residues connected by β, 1⇒4 linkage.

NAG is an important metabolite for *E. coli*. It is not just an excellent source of carbon and nitrogen but also an important constituent of the cell wall and cell membrane. There are many players involved in uptake, catabolism and synthesis of NAG. Two divergent operons, *nagE* and *nagBACD* encode enzymes involved in uptake and catabolism of NAG (Plumbridge, 1991). The gene *nagE* codes for the enzyme II^{nag}, which is a phosphoenolpyruvate (PEP)-dependent permease involved in uptake of NAG. It belongs to the functional superfamily of phosphotransferase system (PTS) sugar transporters. It is located in the inner membrane and transports NAG inside the cell, at the same time phosphorylates it to N-acetyl glucosamine-6-phosphate. NagE transports NAG with a low micromolar affinity, however, plays a significant role in peptidoglycan recycling. The genes *nagA* and *nagB* encode catabolic enzymes. NagA is N-acetyl glucosamine-6-phosphate deacetylase, which catalyzes the first step of catabolism of NAG. NagB is glucosamine-

6-phosphate deaminase, which catalyzes the second step of NAG catabolism. The action of NagB produces ammonia and fructose-6-phosphate, which can enter glycolysis directly. Deletion mutants of *nagA* or *nagB* are unable to survive on NAG as a sole source of carbon. NagC is a DNA-binding transcriptional dual regulator that binds to the intergenic region of *nagE* and *nagB* (figure 4.1). The main inducer of NagC is N-acetyl glucosamine-6-phosphate. NagC is displaced from DNA upon interacting with N-acetyl glucosamine-6-phosphate. The binding of NagC requires formation of a loop of DNA, so that it binds to two symmetric operator elements at the same time and regulates the expression from both *nagE* and *nagBACD* operons (Plumbridge & Kolb, 1998). Apart from NagE, ManX also transports NAG, which is a PTS transporter and functions in a similar way as that of NagE. NagC also binds to upstream region of manX and de-represses manXYZ operon upon binding to N-acetyl glucosamine. NagC binds to several other sites on DNA regulating the expression of many operons.

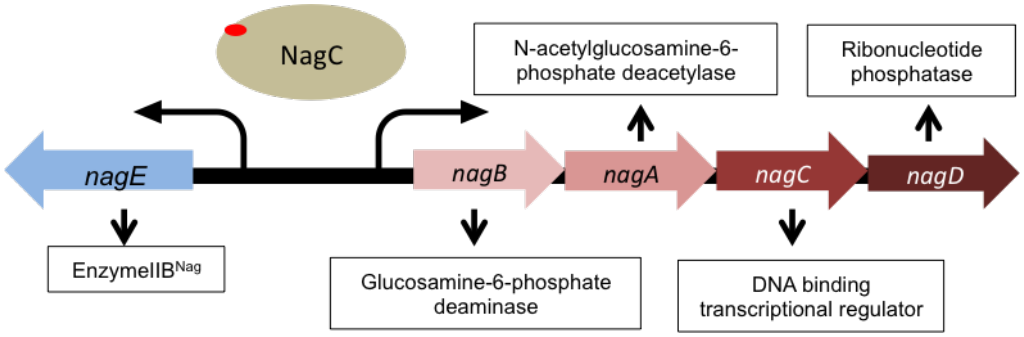

Figure 4.1. – Organization of the *nagE/BACD* operon in *E. coli* (adapted from Plumbridge (1991))

The major source NAG entering the cell is hydrolyzed peptidoglycan. Recycling of peptidoglycan is a routine process that the cell undergoes as it grows and divides. There are several enzymes in the periplasmic space, which hydrolyze peptidoglycan into various intermediate products such as anhydromurapeptides, amino sugars and peptides. The oligopeptide transporter Opp takes up peptides. NagE and other PTS transporters take up amino sugars and AmpG takes up anhydromurapeptides. NagE is responsible for only 50% of N-acetyl glucosamine-6-phosphate generated in the cell. The remaining 50% comes from catabolism of anhydromurapeptides. Enzymes like NagZ, AnmK, MurQ and NagK carry out different steps to generate N-acetyl glucosamine-6-phosphate, which is further catabolized by NagB and NagA (Park & Uehara, 2008).

In addition to catabolizing NAG *E. coli* can also synthesize NAG from other sugars. The genes *glmU, glmS,* and *glmM* encode for enzymes required for NAG synthesis. Out of these genes, *glmU* and *glmS* are located in the same operon, whereas *glmM* is located elsewhere. GlmS is L-glutamine fructose-6-phosphate aminotransferase, which transfers amino group from glutamate to fructose-6-phosphate resulting in formation of glucosamine-6-phosphate and glutamate. This activity is antagonistic to that of NagB. This is the

first step in hexosamine synthesis. GlmM is phosphoglucosamine mutase, which converts glucosamine-6-phosphate into glucosamine-1-phosphate. GlmU is a fused enzyme with two catalytic activities, glucosamine-1-phosphate acetyl transferase and N-acetyl glucosamine-1-phosphate uridyltransferase. The former activity adds acetyl group to glucosamine-1-phosphate and the latter adds UDP moiety to it resulting in the formation of UDP-N-acetyl glucosamine-1-phosphate. Uridylated NAG is further used in synthesis of peptidoglycan and lipoproteins. The expression of the *glmUS* operon is regulated by NagC. However, in this case NagC acts as an activator. In the absence of N-acetyl glucosamine-6-phosphate, NagC binds to upstream sequence of *glmU* and activates its transcription. Upon binding to N-acetyl glucosamine-6-phosphate, NagC is unable to bind to DNA and the transcription of *glmUS* falls by 5 fold (Plumbridge, 1995). Figure 4.2 describes metabolism of NAG in a concise manner.

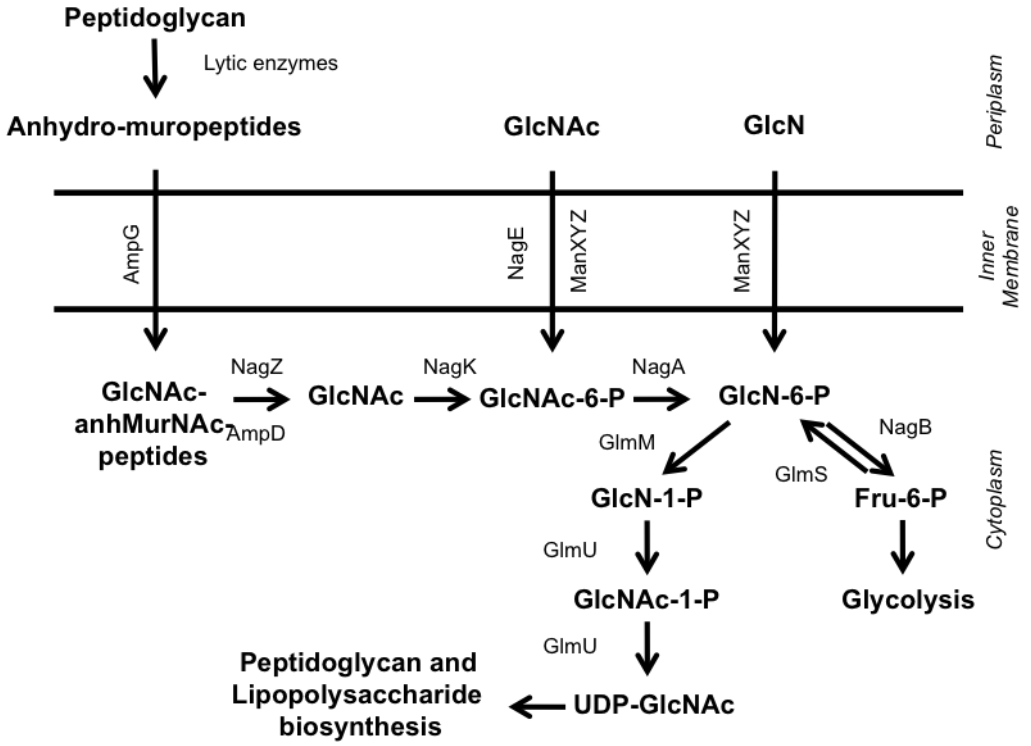

Figure 4.2. – Pathways of NAG metabolism in *E. coli* (adapted from Uehara & Park (2004))

4.2. Chemotaxis in *E. coli*

NAG is not just a metabolite but also a chemoattractant. *E. coli* cells are able to move towards NAG through a phenomenon termed as chemotaxis. Chemotaxis is movement of an organism in response to a chemical stimulus. Many prokaryotes, single celled eukaryotes and certain cells in multicellular organisms exhibit this phenomenon. For bacteria, it is important to move towards a compound that can be taken up as food or to move away from a compound that can be dangerous. Bacterial chemotaxis is one of the most studied

signal transduction pathways. Due to its relative simplicity and modularity it is amenable to manipulations. Chemotaxis can be combined with other signaling pathways to make the engineered bacterium more efficient in its action. Chemotaxis in *E. coli* has been reviewed in great detail (Baker *et al.* , 2006).

4.2.1. Components of the chemotactic pathway

The pathway of chemotaxis in *E. coli* consists of two important sets of players, transmembrane chemoreceptors that sense the chemical stimulus in the environment, intracellular proteins that relay the signal to the flagella and the flagellum (one or many) along with motor proteins that drive flagellar rotation. Chemoreceptors are transmembrane proteins with a highly variable periplasmic sensing domain and a well conserved intracellular signaling domain. In *E. coli* there are 5 types of chemoreceptors, Tar, Tsr, Tap, Trg and Aer. They exist in higher-order array at one or both the cell poles. They are termed as methyl-accepting chemotaxis proteins (MCPs) due to their ability to become methylated. MCPs are responsible for sensing the chemical stimulus in the environment. At the signaling domain of an MCP lies a complex of intracellular proteins including CheA and CheW. CheA is a typical Histidine Kinase, which autophosphorylates and transfers the phosphate group to a Response Regulator CheY. CheW provides a physical coupling of CheA and MCPs and mediates the phosphotransfer between CheA and CheY. CheY is a typical response regulatory, which contains a phosphorylation domain and motor protein-binding domain (instead of a DNA-binding domain present in most response regulators). CheY interacts with motor proteins and influences their direction of rotation. CheZ is an intracellular protein that has phosphatase activity. It dephosphorylates CheY and maintains its level at a steady state. CheR is another intracellular protein located at the signaling domain of MCPs, which methylates glutamate side chains of an MCP. CheB counteracts CheR by de-methylating an MCP.

4.2.2. Signal transduction in chemotaxis

The process of signal transduction in chemotaxis has been reviewed extensively (Eisenbach, 1996; Bren & Eisenbach, 2000; Falke *et al.* , 1997). Figure 4.3 describes how transduction of signal during chemotaxis takes place. In the absence of any attractant, the activity of CheA is stimulated, increasing the phosphotransfer rate from CheA to CheY. Phosphorylated CheY has a lower affinity to CheA and higher affinity to FliM, a component of the motor protein complex. Thus, it dissociates from CheA and binds to FliM, changing its direction of rotation from counter-clockwise to clockwise. When the flagellar motor is rotating counter-clockwise, all flagella coalesce into a bundle, which propels the cell forward (run). Changing the direction of rotation causes the bundle to disperse and the cell to tumble. The movement of a cell is a random walk made up of

alternating runs and tumbles. CheZ prevents over-accumulation of CheY-P by dephosphorylating it and thus maintains its steady-state level generating a random walk. Once an attractant compound binds to the periplasmic sensing domain of an MCP, it undergoes a conformational change and inhibits the kinase activity of CheA. This lowers the rate of phosphotransfer to CheY and drops the levels of CheY-P. This results in lower frequency of tumbling as the cell moves towards higher concentration of the attractant. Adaptive regulation is necessary to prevent an MCP from saturating and enable it to respond to increasing concentration of the attractant. CheR and CheB carry out this adaptation. CheR is a constitutive enzyme that methylates glutamine side chains of an MCP through the use of S-adenosylmethionine. Methylation enhances the kinase activity of CheA. At a steady state, CheA phosphorylates CheB along with CheY. CheB-P counteracts CheR by demethylating the MCP. Thus, kinase activity of CheA is maintained. Upon binding of the attractant to the MCP, kinase activity of CheA is inhibited leading to fall in level of CheB-P. This lowers demethylation and the activity of CheR improves the kinase activity of CheA, thereby resetting the kinase to its basal level. This enables the kinase to respond to increasing concentration of the attractant.

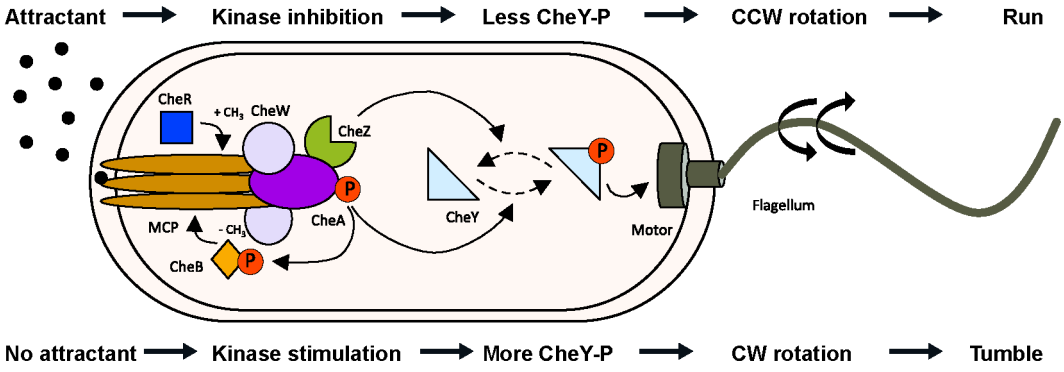

Figure 4.3. – The pathway of chemotaxis in *E. coli* (figure adapted from the PhD thesis of David Kenter, 2010)

4.2.3. PTS-mediated chemotaxis

Apart from this MCP-dependent pathway, there exists an alternative receptor-independent chemotactic pathway that operates through phosphoenolpyruvate sugar phosphotransferase systems (PTS). Mechanistic details of this pathway have been explored in great detail (Lux *et al.* , 1995, 1999; Neumann *et al.* , 2012). A typical PTS involves a membrane bound transporter called Enzyme II, and intracellular proteins Enzyme I and HPr. Enzyme II takes up the sugar from the environment while phosphorylating it. Every sugar is transported through its specific transporter. Enzyme II can be a single multi-domain protein or a group of small proteins. The phosphate group used in this process is received from a relay of phosphate carried out by Enzyme I and HPr. Enzyme I (encoded by *ptsG*) is a PEP-dependent histidine kinase, which takes up the phosphate group from

phosphoenolpyruvate. It transfers the phosphate group reversibly to HPr, a phosphohistidine carrier protein, which eventually transfers it to Enzyme II for sugar uptake. There are 15 such PTSs in *E. coli*, Enzyme I and HPr being common to all. Several sugars are taken up though these systems. *E. coli* exhibits chemotaxis to these sugars since they act as carbon sources. Enzyme I, in its unphosphorylated form binds to CheA and inhibits its kinase activity. This effect is the same as that of an attractant compound binding to the MCP. With increasing concentration of the sugar, levels of unphosphorylated Enzyme I also increase and cause the cell to move with less frequent tumbles. This results in chemotaxis of the cell towards the sugar. As stated earlier, the *E. coli* cell internalizes NAG through PTS. Thus, the chemotactic pathway for NAG is likely to be through the PTS. Figure 4.4 shows a schematic of the PTS-mediated chemotaxis pathway.

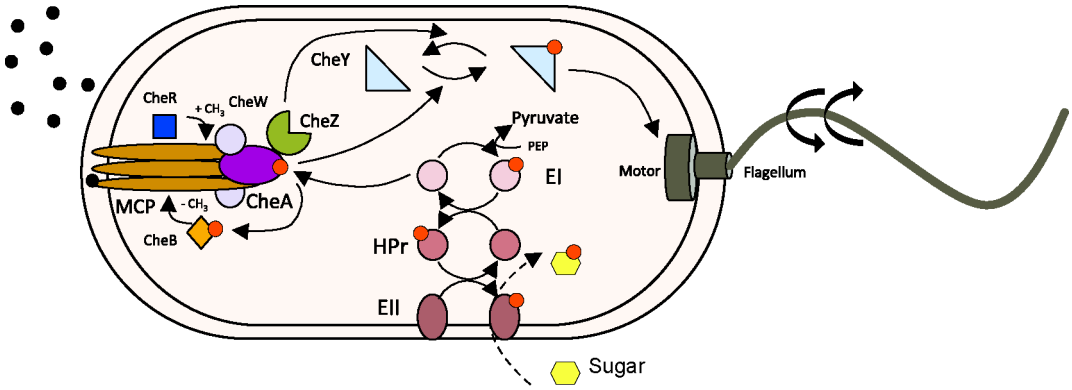

Figure 4.4. – The pathway of PTS-mediated chemotaxis in *E. coli* (figure adapted from Neumann *et al.* (2012))

4.3. Quorum sensing in *S. epidermidis*

Quorum sensing is a system of stimuli and responses correlated to the population density. Many bacteria use this system to coordinate the gene expression according to the density of their local population. Bacteria regulate a diverse array of physiological functions such as motility, sporulation, virulence, conjugation, biofilm formation and many others through the system of quorum sensing (Miller & Bassler, 2001).

There is a single quorum sensing system found in *S. epidermidis*, which is encoded by *agr* (accessory gene regulator) operon (figure 4.5). Much of the knowledge of this system in *S. epidermidis* is based on that in *S. aureus* (Thoendel *et al.* , 2011). The *agr* locus consists of two operons *RNAII* and *RNAIII*. The operon *RNAII* encodes 4 ORFs *agrB, agrD, agrC,* and *agrA*. AgrD is a peptide that is processed and exported by AgrB. The secreted peptide is 8 amino acids long and shows cyclization at the C-terminus (Otto 1998). This peptide acts as signaling molecule. AgrC is a histidine kinase located at the cell membrane. It senses the AIP in the environment and phosphorylates the response regulator AgrA. AgrA then binds to the intergenic region between *RNAII* and *RNAIII* and

activates the expression of both the operons. Activation of *RNAII* leads to more synthesis of AIP. The transcription product of *RNAIII* has a dual role. It mediates the synthesis of deltatoxin and also functions as a regulatory RNA that alters the expression of many other genes.

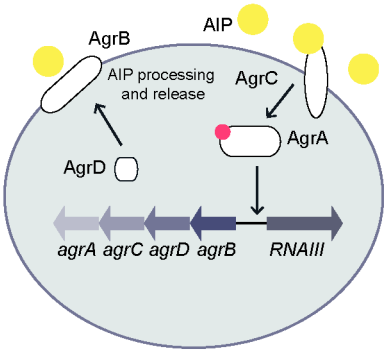

Figure 4.5. – The *agr* quorum-sensing system of *S. epidermidis* (figure adapted from Thoendel *et al.* (2011))

4.4. Protein secretion systems in *E. coli*

Another important aspect of an engineered bacterial system is its ability to secrete a protein of interest. Secretion makes the protein available in the extracellular space for its intended action. Thus, it is necessary to take an overview of protein secretion systems in *E. coli*.

E. coli possesses several different kinds of protein secretion systems. For any protein to be secreted out of the cell, it has to pass through three barriers- inner membrane, periplasm and outer membrane. Depending on the mechanism by which the proteins are transported through these barriers, secretion systems have been classified into 6 different types. The structure, assembly and the mechanism of these systems have been explored well in detail (Gerlach & Hensel, 2007; Kostakioti *et al.* , 2005).

4.4.1. T2SS and the general secretory pathway

Among various secretion systems present in *E. coli*, the type II secretion system (T2SS) is described here. T2SS is the terminal step of the general secretion pathway (steps D1 and D2 in figure 4.6). It consists of multimeric porin complexes in the outer membrane. For proteins to cross the inner membrane, there are two major types of pathways- Sec-dependent and Sec-independent (TAT). There is an important subtype of the Sec-dependent pathway, which is called SRP pathway. These pathways together constitute the general secretion pathway. Figure 4.6 is a schematic representation of the general secretion pathway in *E. coli*.

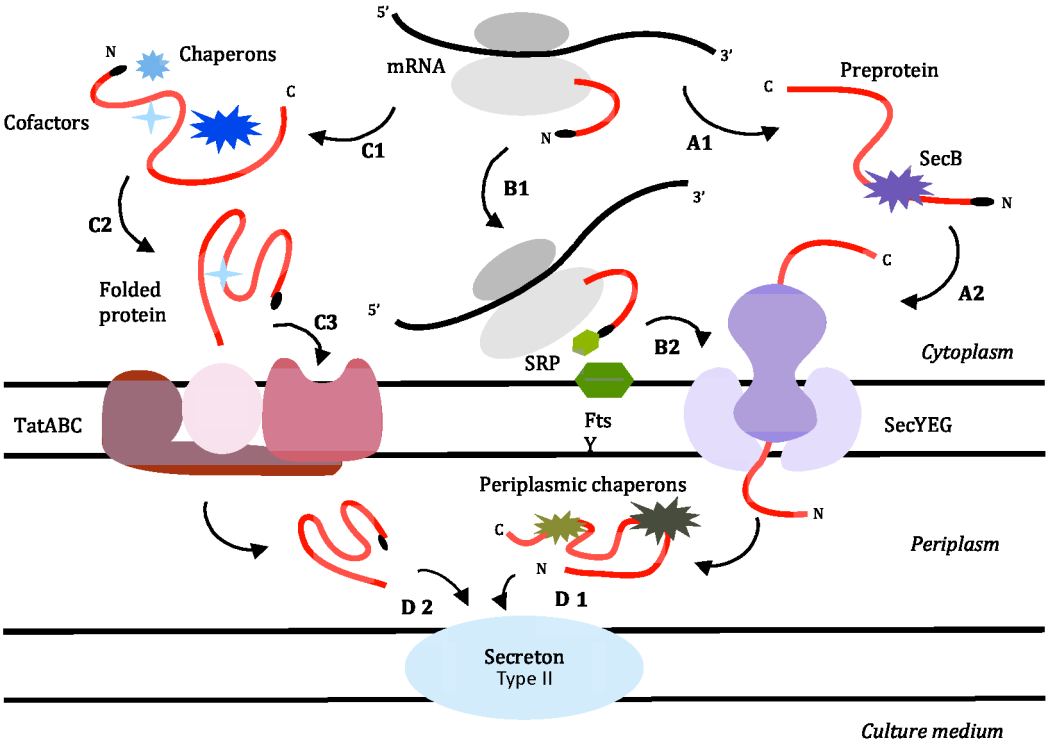

Figure 4.6. – The general secretory pathway of protein secretion in *E. coli* (figure adapted from Mergulhão *et al.* (2005))

In the Sec-dependent pathway, a protein to be secreted is synthesized in the cytoplasm in unfolded or partially folded form. SecA recognizes the preprotein while binding to its N-terminal signal sequence. SecB is a chaperon that maintains it in the unfolded state (step A1 in figure 4.6). It is then brought to SecYEG export complex located in the inner membrane (step A2 in figure 4.6). The preprotein is then exported into the periplasm with the help of many other proteins. Chaperons present in the periplasm help in folding the preprotein into a functional form. In SRP pathway, a signal recognition particle (SRP), similar to the one in eukaryotes, recognizes the preprotein and brings it to SecYEG complex independent of SecA (steps B1 and B2 in figure 4.6). The preprotein is exported out of the inner membrane in a co-translational manner (Beckwith, 2013).

In the Sec-independent pathway, also known as Twin Arginine Translocation (TAT) pathway, proteins are exported in folded form. The protein to be exported is recognized by TatB/TatC complex and other components of TAT pathway assemble at the inner membrane to form a translocation pore. The protein is then exported through this pore (steps C1 and C2 in figure 4.6). The TAT export complex has extraordinary structural flexibility. Proteins exported through TAT system vary greatly in size, structure and function (Lee *et al.* , 2006).

4.4.2. Secretion of heterologous proteins

Engineering *E. coli* cells to secrete heterologous proteins into the surrounding medium offers several advantages over intracellular production. Proteins secreted in the medium can be easily purified due to reduced contamination by other intracellular proteins. The process of secretion also saves the heterologous protein from the action of intracellular proteases. Researchers have explored various strategies to improve secretion of heterologous proteins by *E. coli* and they have been reviewed (Yoon *et al.* , 2010).

Among all the explored strategies, fusing the heterologous protein to a signal sequence is the easiest strategy. The secretions signal sequences of several proteins such as OmpA, PelB, PhoA, YebF, FlgM etc. have been tested so far (Mergulhão *et al.* , 2005). The secretion signal sequence of OmpA has been used extensively for export of many heterologous proteins (Takahara *et al.* , 1985; Guisez *et al.* , 1998).

4.4.3. The protein OmpA and its secretion signal

OmpA is a non-specific diffusion channel located in the outer membrane of *E. coli*, allowing various solutes to pass through. It is the most abundant protein in the outer membrane. It is a multifaceted protein that functions as a porin and an adhesin; it is exploited by bacteriophages for attachment, it mediates F-factor dependent conjugation and is also involved in maintaining morphology of the outer membrane (Smith *et al.* , 2007a). It is 325 amino acids long and first 171 residues form a β-barrel structure with eight amphipathic antiparallel β strands connected by four long loops at the outer surface of the membrane and three at the periplasmic side (Reusch, 2012). The C-terminal portion of the protein resides in the periplasm and perhaps interacts with peptidoglycan (De Mot & Vanderleyden, 1994).

OmpA is exported through the inner membrane via Sec-dependent system. The periplasmic chaperon protein Skp maintains it in unfolded state. It is then incorporated into the phospholipid bilayer of the outer membrane with the help of the Bam outer membrane assembly protein complex (Bulieris *et al.* , 2003). The first 21 amino acid residues at the N-terminus of OmpA serve as a secretion signal (Movva *et al.* , 1980). This stretch of 21 amino acids possesses all the features of a typical secretion signal. It has 2 lysine residues at the N-terminus imparting basic character, a long middle region of hydrophobic residues and a cleavage site around alanine residue. This sequence of 21 amino acids can be fused to heterologous proteins to drive their secretion out of the cell. Figure 4.7 shows the sequence of the secretion signal and the topology of the OmpA protein.

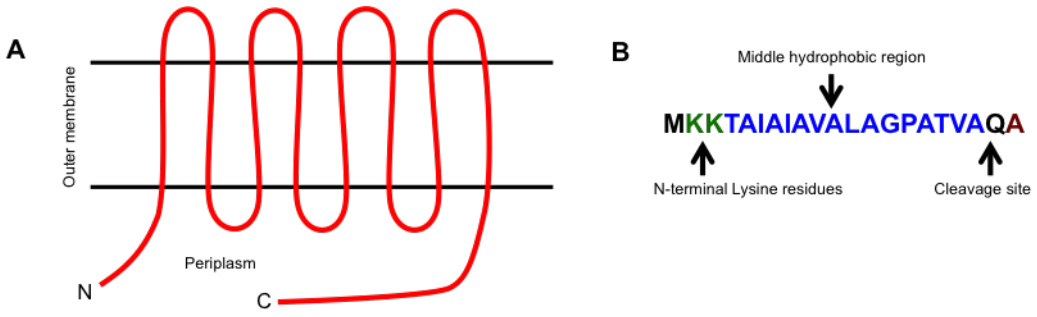

Figure 4.7. – The OmpA protein **A**. Topology of the protein (Smith *et al.* (2007b)), **B**. Sequence of the secretion signal of OmpA (Movva *et al.* (1980))

5. Aims of the current work

The objective of this project is to develop a novel and efficient strategy to combat bacterial biofilms. Figure 5.1 describes the general strategy of this project. The engineered bacterium, regarded as the '*killer*' detects the presence of a target biofilm, regarded as the '*victim*' by sensing a compound specific to the '*victim*'. In response to this detection, the '*killer*' starts synthesizing an anti-biofilm compound, regarded as the '*weapon*'. This '*weapon*' is then secreted and the '*victim*' is disrupted.

To materialize the whole concept, one must divide the objective into different steps. The first step will be to select the '*killer*' and the '*victim*'. The next step will be to select the '*weapon*' expressed by the '*killer*' that efficiently disrupts the biofilm of the '*victim*'. The final and the most important step will be to select a suitable signaling pathway through which sensing ('*victim*' detection) and response ('*weapon*' deployment) can be combined.

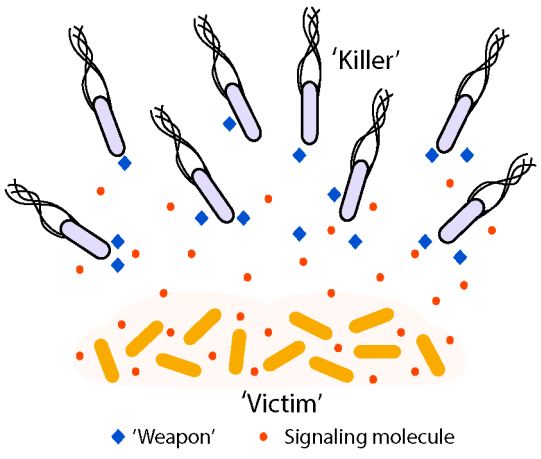

Figure 5.1. – The strategy of engineering bacteria to disrupt a bacterial biofilm

Part II.

Materials and methods

6. Materials

6.1. Buffers

Buffers used in this study are listed in table 6.1.

Buffer	Composition
Phosphate buffer	1.742 g K_2HPO_4 1.361 g KH_2PO_4 Add ddH_2O to a total volume of 1 l and adjust pH to 7
Tethering buffer	1.742 g K_2HPO_4 1.361 g KH_2PO_4 37.2 mg EDTA 10 µl 100 µM L-Methionine Add ddH_2O to a total volume of 1 l and adjust pH to 7
PNAG buffer	8.77 g NaCl 1.21 g TRIS base Add ddH_2O to a total volume of 1 l and adjust pH to 7
TSS buffer	5 g PEG 8000 0.3 g $MgCl_2.6H_2O$ 2.5 ml DMSO Add LB medium to a total volume of 50 ml and sterilize by filtration

Table 6.1. – Buffers used in this study

6.2. Media

Media used for growing bacteria are listed in table 6.2.

Medium	Composition
LB (Luria Bertani) medium	10 g Bacto tryptone 5 g Bacto yeast extract 5 g NaCl Add ddH_2O to a total volume of 1 l and adjust pH to 7
TB (Tryptone broth)	10 g Bacto tryptone 5 g NaCl Add ddH_2O to a total volume of 1 l and adjust pH to 7
TSB (Tryptic soy broth)	30 g dehydrated TSB (Sigma-Aldrich) Add ddH_2O to a total volume of 1 l and adjust pH to 7
5X M9 salts	64 g $Na_2HPO_4.7H_2O$ 15 g KH_2PO_4 2.5 g NaCl 5 g NH_4Cl Add ddH_2O to a total volume of 1 l and adjust pH to 7
M9 minimal medium	200 ml 5X M9 salts 10 ml 20% Glycerol 1 ml 1M $MgSO_4$ 0.5 ml 0.1 M $CaCl_2$ 1 ml 10% Casein hydrolysate Add ddH_2O to a total volume of 1 l and adjust pH to 7
5X minimal A salts	52.5 g K_2HPO_4 22.5 g KH_2PO_4 5 g $(NH_4)_2SO_4$ 2.5 g Na citrate Add ddH_2O to a total volume of 1 l and adjust pH to 7
Minimal medium A agar	200 ml 5X Minimal A salts 10 ml 20% Glycerol 1 ml 1M $MgSO_4$ 8 ml 5mg/ml amino acid mix (L- methionine, L- cysteine, L- threonine, L- histidine) 2 ml 50 mg/ml thiamine 500 ml 0.45% agar (dissolved in sterile ddH_2O) Add ddH_2O to a total volume of 1 l and pour into Petri plates

Table 6.2. – Media used in this study

To make solid media, agar was added to the respective medium to make a final concentration of 1.5%. Unless and otherwise stated, all media were sterilized by autoclaving at 15 psi, 121-124 °C for 15-20 minutes.

6.3. Antibiotics

Antibiotics used in this study are mentioned in table 6.3. All antibiotics were purchased from Sigma-Aldrich. Stock solutions were prepared in suitable solvents and stored at -20

°C. Stock solution was thawed and diluted to working concentration as per the requirement.

Antibiotic	Stock concentration	Working concentration	Solvent
Ampicillin	100 mg/ml	100 µg/ml	ddH_2O
Kanamycin	50 mg/ml	50 µg/ml	ddH_2O
Chloramphenicol	100 mg/ml	50 µg/ml	Ethanol

Table 6.3. – Antibiotics used in this study

6.4. Reagents used in molecular biology techniques

Enzymes used for amplification and manipulation of DNA, were purchased from New England Biolabs, Thermo Scientific, and Invitrogen. Size standards for DNA, RNA and proteins and dyes for their visualization were purchased from Invitrogen.

6.5. Kits

Routine protocols of molecular biology such as plasmid isolation, agarose gel extraction and purification, RNA isolation and also protein purification were performed with the help of commercially available kits. Table 6.4 enlists the kits that were used in this study.

Name of the kit	Application	Company
GeneJET Plasmid mini-prep kit	Plasmid isolation	Thermo Scientific
GeneJET Gel extraction kit	Extraction of DNA fragment from agarose gel and its purification	Thermo Scientific
GeneJET PCR purification kit	Purification of amplified DNA from PCR reaction mixture	Thermo Scientific
Universal RNA extraction kit	Isolation of total RNA	Roboklon
Protino NI-IDA 2000 packed columns	Purification of His-tagged proteins	Machery-Nagel

Table 6.4. – Kits used in this study

6.6. Softwares

Following is the list of softwares used for different analyses and visualization (Table 6.5).

Name of the software	Application
Serial cloner	*In-silico* DNA manipulation
ArrayStar by DNAStar	Analysis of RNA-seq data
Kaleidagraph	Graphical representation of quantitative data
FlowJo	Analysis of flow-cytometry data

Table 6.5. – List of softwares used in this study

7. Methods

7.1. Growth and cultivation of bacteria

Bacteria were mostly grown in Luria Bertani (LB) medium supplemented with appropriate antibiotics. In some cases, Tryptone broth or M9 minimal medium was used. For long-term storage, overnight culture of a bacterial strain was spun down at 10000 rpm in a table-top centrifuge and re-suspended in LB mixed with 20% Glycerol. Afterwards it was stored at -80 °C. For short-term storage, bacterial strains were streaked out on LB-agar plate (containing appropriate antibiotics), grown for 16 to 20 hours at 37 °C and then stored at 4 °C. For performing an experiment, a tiny portion of a bacterial colony from an LB-agar plate or a little frozen cell mass scratched off from the frozen bacterial glycerol-stock was inoculated in LB medium and grown overnight (12 to 16 hours) at 37 °C on a culture-wheel. This overnight culture was used for the experiments as described in respective protocols.

7.2. Molecular biology techniques

Molecular biology techniques used in this study were performed using standard protocols as described in the book 'Molecular Cloning- a laboratory manual' (Green, 2012). Manufacturer's instructions were followed wherever commercially available kits were used. Making of certain constructs used in this study is further described more in the detail.

7.2.1. Plasmid isolation

The isolation of plasmid DNA was performed using GeneJET plasmid mini-prep kit (Thermo Scientific).

7.2.2. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify the desired fragment of DNA from genomic DNA or plasmid DNA as a template. The enzymes used for amplification were purchased from Thermo Scientific. 'Phusion High Fidelity DNA Polymerase' (catalog number) was mainly used amplifying for fragments larger than 1000 bp. and T4 DNA

Polymerase (for DNA fragments smaller than 1000 bp) both purchased from Thermo Scientific. PCR was also used to fuse two different fragments of DNA into one fragment. The technique is described as Overlap Extension PCR (Horton *et al.* , 1989). After setting the reaction mixture, amplification was carried out in peqSTAR 96X Thermocycler. A variant of PCR, called colony-PCR was used to screen for correct recombinants. A small portion of the bacterial colony was used instead of a genomic or plasmid DNA as a template and amplification was carried out as described above. Successful amplification of the DNA fragment was confirmed by visualizing the product of the reaction on an agarose gel. The reaction mixture was purified using GeneJET PCR purification kit to purify the amplified DNA for further use.

7.2.3. Restriction digestion

DNA fragments or plasmids were digested by restriction endonucleases purchased from New England Biolabs. Manufacturer's protocol was followed to set up the digestion reaction. The digested DNA fragment or plasmid was purified with the help of GeneJET Gel Extraction kit or GeneJET PCR-purification kit, both purchased from Thermo Scientific.

7.2.4. Ligation

Digested DNA fragments were ligated into digested plasmids (cloning vectors) by using T4 DNA Ligase enzyme purchased from Thermo Scientific. A ligation mixture was set in such a way that insert and vector were in 3:1 or 9:1 ratio and the total amount of DNA was at least 50 ng in the total volume of 10 µl. The mixture was incubated at room temperature for around 10 minutes and used for transformation.

7.2.5. Preparation of chemically competent *E. coli* cells

$CaCl_2$ method *E. coli* cells were grown to OD_{600} of approximately 0.6. The cells were spun down (10 min, 4000 rpm, 4 °C) and re-suspended in 0.1 M $MgCl_2$ so that the volume is half of the original culture. The cells were incubated on ice for 30 min and spun down again under the same conditions. Re-suspension was carried out with 0.1 M $CaCl_2$ so that the volume remains the same as previous step. The cells were incubated on ice for 5 minutes and spun down again under the same conditions. The pellet was re-suspended in 0.1 M $CaCl_2$ with 18% glycerol in such a way that the total volume is 1/10th of the original culture. This re-suspension was directly used for transformation or frozen using liquid nitrogen and stored at -80 °C.

TSS method *E. coli* cells were grown to OD_{600} of approximately 0.6. The cells were spun down at maximum speed in a tabletop centrifuge. The pellet was re-suspended in

TSS buffer so that the volume is 1/10th of the original culture. The re-suspension was incubated on ice for at least 30 minutes and used for transformation. Alternatively, it was frozen using liquid nitrogen and stored at -80 °C for further use.

7.2.6. Transformation

To introduce plasmids into *E. coli* cells, 100 µl of chemically competent cells were thawed on ice. The desired plasmid or a ligation mixture was mixed with the competent cells and incubated on ice for 30 minutes. The mixture was given a heat shock at 42 °C for 45 seconds followed by incubation on ice for 2-3 minutes. The cells were allowed recover by adding pre-warmed LB medium and shaking at 37 °C for approximately one hour. This mixture was then spun down at high speed in a tabletop centrifuge and plated on an LB-agar plate containing respective antibiotic. The plate was incubated at 37 °C for 16 to 20 hours for colonies to appear. Colonies observed on this plate were re-streaked on a fresh LB-agar plate. These re-streaked colonies were used to perform colony-PCR for screening for the correct recombinant.

7.2.7. Agarose gel electrophoresis

Mixtures containing DNA to be visualized were mixed with 6X DNA loading dye (purchased from Thermo Scientific) and loaded on 1% agarose (in TAE buffer) gel slab mixed with Midori Green (purchased from Nippon Genetics Europe GmBh). Midori Green is a fluorescent nucleic acid stain, which shows strong fluorescence upon binding with DNA or RNA. The fragments of DNA were separated under constant voltage of 120 V for approximately 30 minutes. Visualization and image acquisition was performed with the help of Del-Doc, a UV Transilluminator by BioRAD.

7.2.8. DNA sequencing

All the constructs made during this study were sequenced at MWG Eurofins GmBh Germany. The samples were prepared according to the instructions provided by the company. Sequencing results were compared with the expected sequence to check the correctness.

7.2.9. Deletion of non-essential genes of *E. coli* by P1 transduction

The library of single-gene knockout-mutants of *E. coli* K12 (Keio Collection) was obtained from GenoBase (Baba *et al.* , 2006). This collection contains 3985 single-gene deletion mutants of non-essential genes of *E. coli* in a common strain background, *E. coli* K12 BW25113. Candidates from this collection were used to transfer the deletion mutation

to the strain commonly used in this thesis, *E. coli* K12 W3110. Overnight culture of the donor (deletion mutant from Keio collection) was diluted 1:100 in 2 tubes of 5 ml of LB medium containing 0.2% glucose and 5 mM $CaCl_2$ (one tube acts as a control). After few minutes of incubation at 37 °C, 100 µl of P1 starter lysate was added to one of the tubes. Both the tubes were incubated on a rotary shaker at 37 °C to allow growth of the cells. After 3 hours, the clear lysate from the experimental tube was filtered with 'Stericup 0.2 micron filter' to get rid of the living cells. Meanwhile, 1 ml of the overnight culture of the recipient strain was spun down at maximum speed and the pellet was re-suspended in P1 buffer (10 mM $MgSO_4$, 5 mM $CaCl_2$). It was then incubated at room temperature for at least one hour (or until the lysate of the donor was ready). Once the lysate was ready, 100 µl of lysate was mixed with the equal volume of pre-treated recipient cells and incubated for 30 min at 37 °C. Afterwards, 1 ml of LB containing 10 mM sodium citrate was added to the mixture and incubated further for 30 min at 37 °C. Cells were then spun down and re-suspended in 100 µl of 1 M sodium citrate and plated on LB-agar plates containing 10 mM sodium citrate and respective antibiotic to select the recombinant cells. These plates were incubated at 37 °C for around 20 hours. The colonies obtained on these plates were screened for the correct recombinant by colony PCR. Correct recombinants were re-streaked on LB-agar plates containing respective antibiotic to obtain a pure strain.

7.3. Purification of Dispersin B

The plasmid pJK618, which contained the CDS of Dispersin B was a gift from Dr. Jeffrey Kaplan, Department of Oral Biology, New Jersey Dental School, Newark, USA (Kaplan *et al.* , 2003) . The CDS of Dispersin B was amplified from pJK618 and cloned in the vector pQE60 to generate the construct pVG22. This construct contained 10X Glycine linker and 6X histidine tag at the C-terminus of Dispersin B.

An overnight culture of *E. coli* M15 harboring the construct pVG22 was diluted 1:100 in one liter of LB medium and grown at 37 °C, 110 rpm until the culture had reached OD_{600} of approximately 0.6. The expression of the enzyme was induced by the addition of 100 µM Isopropyl β-D-1 thiogalactopyranoside (IPTG). Expression was carried on for 4 hours under the same conditions. The grown culture was centrifuged at 6000 rpm for 20 minutes at 4 °C. The supernatant was discarded and the pellet was stored at -20 °C for further use. For purification of Dispersin B, NI-IDA 2000 packed columns for purification of His-Tag proteins from Machery-Nagel was used. The protocol for purification was performed as mentioned in the accompanying user manual. The frozen pellet was thawed on ice and re-suspended in 24 ml of 1X LEW buffer (provided in the kit). Lysozyme (purchased from Sigma-Aldrich) was added to the suspension, having the final concentration of 1 mg/ml. A tablet of Roche protease inhibitor was also added to this suspension. The suspension was incubated on ice and was gently shaken on Heidolph gel rocker for 30 min at 4 °C.

This was followed by sonication of the cell suspension (5 pulses of 30 s each at 50% amplitude, 1 s on and 1s off, separated by cooling on ice for 1 min). Lysed cells were centrifuged at 10000 rpm for 25 min at 4 °C. The supernatant (cell lysate) was carefully separated and loaded on the Ni-IDA packed column pre-equilibrated with 1X LEW buffer. The lysate was allowed to flow through the column matrix by gravity. The column was washed thrice with the same 1X LEW buffer. At last, the bound protein was eluted by adding 1X elution buffer in 3 fractions. About 90% of the protein was found in the first fraction. However, all three elution fractions were pooled together and concentrated using Amicon centrifugal filter units (MWCO 10 kDa, purchased from Sigma-Aldrich) at 4000 rpm for 15 min at 4 °C. The resulting concentrate was sorted in 100 µl aliquots with 5% glycerol and flash-frozen with liquid nitrogen. Frozen protein was stored at -80 °C for future use.
All fractions of the process of purification were analyzed by SDS-PAGE (Sodium dodecyl sulfate- Polyacrylamide Gel Electrophoresis) (LAEMMLI, 1970).

7.4. Assay to measure the activity of Dispersin B

To measure the activity of purified Dispersin B, a simple assay was performed as described previously (Shibata & Yagi, 1996). The substrate 4-nitrophenyl N-acetyl-beta-D-glucosaminide (NP-GlcNAc) was dissolved in phosphate buffer to make a solution of 1 mM. Ten microliters of the purified enzyme was added to 1 ml of this solution and it was incubated at 37 °C for 15 min. The reaction was stopped by adding 5 µl of 40% NaOH. The intensity of the color was measured by determining the optical density of the reaction mixture at 400 nm with the help of a Tekan Infinte M1000 Pro microplate reader.

7.5. Dispersin B secretion assay

An overnight culture of *E. coli* M15 harboring the construct pVG21 was diluted 1:100 in M9 minimal medium with glucose and casein hydrolysate as a carbon source. The medium also contained 1 mM of NP-GlcNAc. The cells were pipetted in a 96-well microplate and grown for 24 hours at 26 °C. After incubation the change in the color of the medium was observed and the activity of the secreted enzyme was measured as described above.

7.6. Growing and analyzing *E. coli* biofilms

The protocol for growing and analyzing biofilms was adapted from the one published previously (O'Toole, 2011). An overnight culture of the relevant strain of *E. coli* was diluted 1:1000 in LB medium and dispensed as 200 µl per well in Costar 96-well microplate (Cat-

alog number 3585, purchased from Sigma-Aldrich). The plate was sealed with Parafilm to avoid evaporation. It was incubated at 26 °C for a time interval depending on the experiment. At the end of the incubation, the planktonic cells in the wells were removed by gentle pipetting. The wells were washed thrice with phosphate buffer and the plate was left on the bench for drying. The dried plate was then stained with 1% crystal violet for 30 min and subsequently washed with distilled water until there is no stain seen in the wash-water. The plate was left on the bench for drying. The stained and dried plate was then de-stained using 70% ethanol for 30 min at room temperature. The plate was gently shaken in a gel rocker for proper dissolution of the stain. Hundred microliters of the dissolved stain from each well was pipetted in a new plate. The intensity of the stain was measured by determining the optical density of the solution at 590 nm with the help of Tekan Infinite M1000 Pro microplate reader. The intensity of the stain dissolved in ethanol correlates to the biofilm formation.

7.7. Experiment to check the effect of Dispersin B on *E. coli* biofilms

The biofilms of concerned *E. coli* strains were grown as described above. Planktonic cells were gently removed and purified Dispersin B diluted in phosphate buffer was added to the pre-formed biofilm. The plate was incubated at 37 °C for one hour. At the end of the incubation, the plate was washed; stained and de-stained to measure the intensity as described above.

7.8. Experiment to check the effect of Dispersin B secreting *E. coli* cells on the biofilm of *E. coli csrA* mutant

The csrA mutant of *E. coli*, TRMG1655 was a gift from Dr. Tony Romeo. The biofilm of *E. coli* TRMG1655 was formed in Costar 96-well microplate for 24 hours at 26 °C. Overnight cultures of *E. coli* K12 W3110 harboring the constructs pVG30 and pVG29 respectively were diluted 1:1000 in LB medium containing suitable antibiotics and were pipetted on the pre-formed biofilm after removing the planktonic cells. The expression of Dispersin B was induced by adding IPTG at varying concentration. The plate was further incubated at 26 °C for varying intervals of time. At the end of this incubation, the plate was washed, stained and de-stained to quantify the biofilm formation as described above.

7.9. Analysis of the activity of *nagE* promoter

The strain of *E. coli* K12 W3110 containing plasmid pVG18 was grown overnight in LB along with a control strain containing empty cloning vector pUA66. The overnight culture was diluted 1:50 in M9 minimal medium containing glycerol as a carbon source. The culture was distributed as 1 ml per well in Greiner 24-well flat bottom microplate (catalog number 662102). Expression from *nagE* promoter was induced by adding 1, 5, 10 and 50 micromolars of N-acetyl glucosamine (purchased from Sigma-Aldrich). This culture was grown inside Tekan Infinite 1000 Pro microplate reader at 37 °C and 168 rpm (double orbital shake) for 3 hours. The growth of the cells (OD_{600}) and fluorescence (excitation 488 nm, emission 510 nm) were monitored at an interval of 15 minutes. In another experiment to assess the same activity, the culture in the same plate was grown at 37 °C, 110 rpm for 3 hours in an incubator-shaker. The culture from each well was diluted in 1:100 in phosphate buffer. The fluorescent intensity of the cells was measured by BD FacsCanto II flow cytometer.

7.10. Preparation of the biofilm supernatant

The overnight culture of *E. coli* TRMG1655 was diluted 1:1000 in 20 ml of LB medium and poured into a sterile plastic Petri dish (Sarstedt 92x16 mm with cams, catalog number 82.1473.001). It was then sealed with parafilm and incubated at 26°C without shaking for 24 hours. At the end of the incubation time, planktonic cells were gently removed; attached cells were scratched with a sterile spreader and re-suspended in 1 ml of phosphate buffer. It was then treated with purified Dispersin B (~500 µg/ml) by shaking at 37 °C, 110 rpm in an incubator-shaker for one hour. The suspension was then passed through a syringe filter (Sartorius, Minisart SRP25, pore size 0.45 µm) to get rid off all the cells. This filtered supernatant was then used for estimation of NAG and testing the activity of *nagE* promoter.

7.11. Preparation of the *Staphylococcus epidermidis* biofilm supernatant

The experiments with *S. epidermidis* were performed in the laboratory of Dr. Jochen Wiesner, LOEWE center for Insect Biotechnology and Bioresources, Fraunhofer-institute für Molekularbiologie und Angewandte Oekologie, Giessen, Germany. An overnight culture of *S. epidermidis* RP62A (ATCC 35984) was grown in Tryptic Soy broth (TSB). The overnight culture was diluted 1:1000 in TSB and dispensed in 10 Petri plates, 20 ml each. The Petri plates were sealed with Parafilm and incubated at 37 °C for 24 hours. After the

incubation period, planktonic cells were discarded, 2 ml of PNAG buffer was added to each plate and the attached cells were scratched off with the help of a spreader. Scratched cells from all the plates were pooled together and the suspension was vortexed thouroughly to separate the clumps. The same suspension was sonicated in the sonication water bath (10 cycles of 1 min each, maximum output). It was then centrifuged at 4000 rpm for 10 min. The supernatant was passed through a syringe filter (Sartorius, Minisart SRP25, pore size 0.45 µm) to get rid off all the cells. The filtrate was frozen in liquid nitrogen and brought to Marburg on dry ice. This filtrate was treated as the biofilm supernatant analogous to that of *E. coli*.

7.12. MBTH assay for estimating NAG

The estimation of NAG in the biofilm supernatant (prepared as above) was performed by MBTH assay (Smith & Gilkerson, 1979). The assay was performed in small glass tubes with metal caps. The total volume of the sample was made to 200 µl, having 0.5 M HCl as a final concentration. The sample was heated in a water bath at 110 °C for 2 hours. After cooling to room temperature in a fume hood, 400 µl of 2.5% $NaNO_2$ was added. The sample was mixed well by vortexing and incubated at room temperature for 5 min. This was followed by addition of 200 µl of 12.5% ammonium sulfamate. The sample was again vortexed and incubated at room temperature for 5 min. After this, 200 µl of 0.25% 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH reagent, purchased from Sigma-Aldrich) was added. The sample was incubated at 37 °C for 30 min. Two hundred microliters of 0.5% $FeCl_3$ was added to the sample and it was incubated again at 37 °C for 5 min. Finally, the reaction mixture was pipetted in a 96-well microplate (Costar catalog number 3770) and its absorbance at 650 nm was measured in Tekan Infinite M1000 Pro microplate reader. The same procedure was also performed with NAG (purchased from Sigma-Aldrich) having final concentration of 1 and 5 µM. The entire assay was performed in duplicates.

7.13. Analyzing the response of *nagE* promoter to the biofilm supernatant

The culture of *E. coli* W3110 cells harboring the construct pVG18 was grown in a 24-well microplate exactly as described above. One hundred microliters of Dispersin B-treated biofilm supernatant was added in one of the wells. The activity of *nagE* promoter was measured by flow cytometry as indicated above.

7.14. Analyzing the activity of *nagE* promoter in the vicinity of *E. coli* TRMG1655 biofilm

The overnight culture of *E. coli* TRMG1655 was diluted 1:1000 in LB medium and pipetted in Costar 24-well microtiter plate (catalog number 3527), one ml in each well. The plate was incubated at 26 °C for 24 hours without shaking to allow the formation of a biofilm. An overnight culture of *E. coli* W3110 harboring the construct pVG18 was diluted 1:100 in M9 minimal medium and grown at 37 °C, 200 rpm for 3 hours. Five hundred microliters of this culture were pipetted gently on top of the pre-formed biofilm of *E. coli* TRMG1655 after removing the planktonic cells. Ten microliters of purified Dispersin B (final concentration 60 µg/ml) were added to the same culture and the plate was incubated at 37 °C for one hour. After the incubation, attached cells were scratched with the help of a pipette-tip and re-suspended in phosphate buffer. The suspension was then used to measure the intensity of fluorescence by flow cytometry.

7.15. Testing the activity of *agr* quorum-sensing system in *E. coli*

E. coli W3110 harboring the constructs pVG47.1 and pVG48.2 was grown overnight in LB medium supplemented with suitable antibiotics. The overnight culture was diluted 1:1000 in M9 minimal medium and grown in the presence of 10 µM IPTG and 10 µg/ml of AIP (autoinducing peptide). The AIP was synthesized by EMC microcollections, Tuebingen, Germany according the protocol published previously (Otto *et al.* , 1998). After growing the cells for 3 hours at 37 °C and 200 rpm, the activity of *agr* system was quantified by flow cytometry.

7.16. Testing chemotaxis on a soft agar plate

Soft agar plates were prepared by pouring minimal medium A containing 0.225% agar in standard Petri plates. After the agar was solidified, the compound to be tested for chemotaxis (10 µl) was spotted at the center of the plate. It was allowed to diffuse in the agar by incubating the plate at 4 °C for 12 to 16 hours. An overnight culture of a bacterial strain to be tested was grown in Tryptone broth (TB) and spotted at a distance of 2 cm from the spot of the compound. Plates were further incubated at 30 °C for 24 hours. After sufficient incubation, plates were photographed for the record.

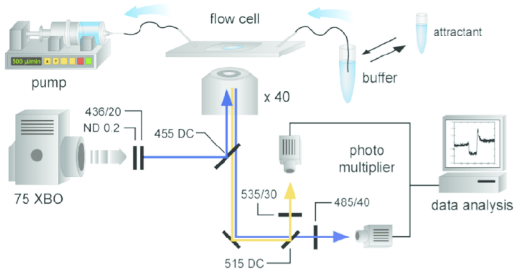

Figure 7.1. – Setup of stimulus-dependent FRET (Dr. Karin Groesse, PhD thesis, 2013)

7.17. Stimulus dependent FRET

Stimulus dependent FRET measurement of the activity of chemotaxis pathway was done with by using CheY-YFP and CheZ-CFP reporter pair as described previously (Sourjik *et al.* , 2007; Kentner & Sourjik, 2009). Figure 7.1 describes the setup of the technique. The same setup was used to quantify chemotaxis of *E. coli* towards N-acetyl glucosamine. An overnight culture of the bacterial strain to be tested was diluted 1:100 in fresh TB medium containing suitable antibiotics and 50 µM of IPTG and grown to OD_{600} of approximately 0.6 at 34 °C and 200 rpm. Cells were then spun down and re-suspended in tethering buffer and incubated at 4 °C for at least 30 minutes. One milliliter of cells were spun down at high speed, re-suspended in 100 µl of tethering buffer and used for FRET measurement.

Polylysine-coated coverslips were prepared by adding 20 µl of polylysine and incubating for 20 min, followed by washing with distilled water. Cells prepared for FRET measurement were added on the coverslip, allowed to attach to polylysine and put into the flow chamber of the flow cell. The flow chamber was maintained at a constant flow rate of 300 µl/min with the help of a syringe pump (Harvard apparatus 22). The flow was stopped briefly to add and remove the attractants. The flow-cell was mounted on an inverted microscope (Zeiss Axiovert 200) and cells were focused under 40X magnification. The fluorescence intensities of YFP and CFP were measured and FRET was calculated from the changes in their ratio as described previously.

Dose-response measurement was performed by stimulating buffer-adapted cells with the addition and the removal of the attractant in increasing concentration. Dynamic range measurement was performed by stimulating attractant-adapted cells with increasing concentration of the attractant. In both the measurements, response to 100 µM of methyl aspartate was used as a control.

7.18. Growing biofilms for RNA isolation

E. coli W3110 WT and its *fliC* deletion mutant were used for studying biofilm transcriptomics. Biofilm was grown in by diluting overnight culture 1:1000 in 20 ml of LB and incubating it for 24 h without shaking at 26 °C in a standard Petri plate. After incubation, planktonic cells were taken out by decanting the medium slowly. Cells attached to the surface of the Petri plate were scratched with the help of a spreader and re-suspended in phosphate buffer. For *ΔfliC* strain, only planktonic cells were harvested assuming there are negligible attached cells. Both planktonic and attached cells were spun down and pellet was stored at – 20 °C.

7.18.1. RNA isolation

Stored pellets of bacterial cells were thawed on ice and re-suspended in TE buffer. Total RNA was isolated from cells using EURX Universal RNA Isolation kit by following manufacturer's instructions.

7.18.2. RNA sequencing

The isolated RNA was submitted to Genomics Core Facility at EMBL Heidelberg.

Part III.

Results

8. Strategy

The objective of this thesis is to design an engineered bacterial system that can efficiently disrupt a bacterial biofilm. The strategy to accomplish this task involves the following steps.

8.1. Choosing the organism to be engineered as the '*killer*'

Escherichia coli is the most well studied bacterium. Several systems involving genetic engineering have been successfully developed using *E. coli* as a chassis. Many signaling pathways in *E. coli* have been characterized (chapter 4). The pathways of protein secretion are also well studied (section 4.4). It is convenient to handle *E. coli* in a laboratory setting. Thus, I chose *E. coli* to design the system planned in this project. Henceforth, the '*killer*' will refer to the strain of *E. coli* that is being engineered for the planned system.

8.2. Choosing the target biofilm that would be the '*victim*'

A great deal of information is known about biofilms of different bacteria, and biofilms of *E. coli* are among the most commonly studied. The composition of the matrix of *E. coli* biofilms is well characterized (section 2.1). Considering the ease of handling *E. coli* in the laboratory, I chose *E. coli* as the target biofilm to demonstrate the action of the planned system.

Alternatively, I also considered *Staphylococcus* epidermidis as a '*victim*'. *S. epidermidis* is a nosocomial pathogen that makes notorious biofilms on indwelling medical devices. A great deal of information is known about the composition of its biofilm matrix (section 1.3.2). Developing an engineered bacterial system against *S. epidermidis* biofilms may have applications in the healthcare sector.

8.3. Choosing a suitable 'weapon' to disrupt the target biofilm

Researchers have explored several methods to combat biofilms (chapter 3). The focus of this project is biological methods. The 'weapon' to be chosen must be compatible with an engineered bacterial system. As described earlier (section 3.4), matrix-degrading enzymes are excellent tools that can efficiently disrupt a target biofilm. Among various enzymes known so far, Dispersin B has been shown to be effective against *E. coli* and *S. epidermidis* biofilms. Its synthesis involves just one step, and it is non-toxic to *E. coli*, allowing for easy overexpression. It can also be easily secreted into the surrounding medium by *E. coli* cells by exploiting known protein secretion pathways. I chose Dispersin B as the 'weapon' in the system planned in this project.

8.4. Choosing a suitable signaling pathway

The most important aspect of this project is to choose the right signaling pathway. The signaling pathway must be able to detect the presence of the '*victim*' and drive the synthesis of the 'weapon' in response to it. I have proposed three different strategies to accomplish this goal.

8.4.1. Using the N-acetyl glucosamine metabolic pathway

Biofilms of *E. coli* contain PGA (poly N-acetyl glucosamine) as one of the components of the matrix. Dispersin B degrades PGA into its constituent monomers, N-acetyl glucosamine (NAG). NAG is a small molecule that can easily diffuse in the surrounding medium. It is a metabolite as well as a chemoattractant. *E. coli* possesses robust signaling and metabolic pathways through which, it detects NAG present in the surrounding medium, imports it, and metabolizes it. Thus, NAG is a highly suitable signaling molecule, and the pathway associated with its metabolism is a fitting signaling pathway for this project. The proposed system is described in figure 8.1. The '*victim*' biofilm possesses PGA and is likely to possess some free NAG as a result of the spontaneous degradation of PGA. The '*killer*' detects NAG and expresses Dispersin B in response to it. Dispersin B, secreted from the '*killer*' degrades PGA present in the '*victim*' biofilm and disrupts it. The action of Dispersin B releases more NAG, which acts as an inducer for further synthesis of Dispersin B. The synthesis of Dispersin B continues as long as there is sufficient supply of NAG.

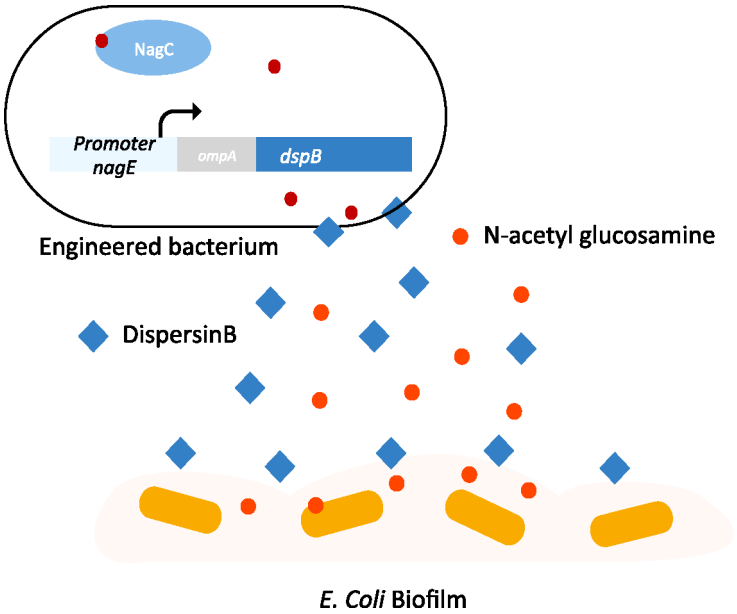

Figure 8.1. – Design of the proposed system that exploits NAG metabolic pathway

8.4.2. Using biofilm-specific gene expression

The pattern of gene expression within a biofilm can be used as a sensor, in which development of biofilm is a response to the biofilm mode of lifestyle. The coordinated expression of certain genes occurring in biofilms is indeed an attractive concept, which can be exploited in the planned system. In this system, the '*killer*' possesses a construct, in which Dispersin B is expressed from a promoter associated with a biofilm-specific gene. As the '*killer*' approaches the '*victim*' biofilm, it acquires the biofilm-lifestyle and gets incorporated into the '*victim*' biofilm. The 'killer' then begins to produce and secrete Dispersin B from the biofilm-specific promoter. Dispersin B, secreted from the '*killer*' degrades PGA in the '*victim*' biofilm and disrupts the biofilm from within. Figure 8.2 describes this concept.

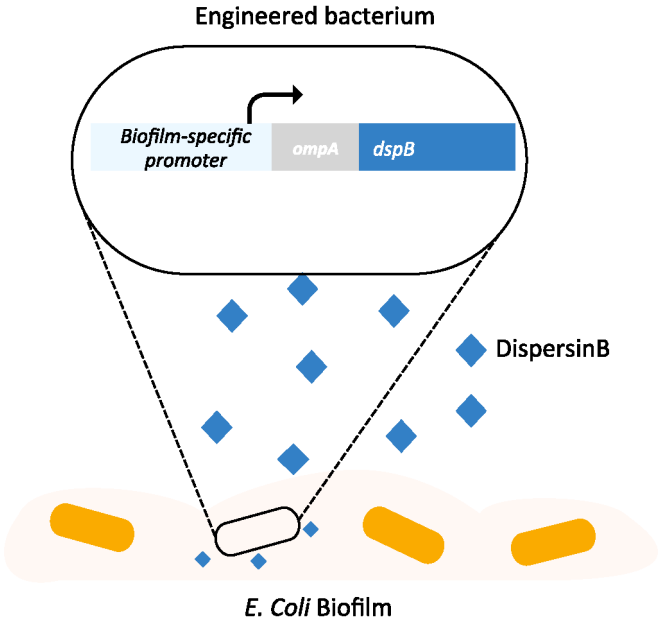

Figure 8.2. – Design of the proposed system exploiting the biofilm-specific gene expression

8.4.3. Using *agr* quorum-sensing system of *S. epidermidis*

S. epidermidis possesses a quorum-sensing system, termed *agr* (accessory gene regulator), that regulates the expression of *RNAII* and *RNAIII* operons through a signaling molecule called AIP (auto-inducing peptide). I plan to exploit this signaling system to disrupt *S. epidermidis* biofilms. In this particular strategy the '*victim*' biofilm is that of *S. epidermidis* and the '*killer*' is the engineered *E. coli*. In this system, the '*killer*' possesses a construct, in which Dispersin B is expressed from an *RNAIII* promoter. It also expresses AgrC and AgrA proteins of *S. epidermidis*. The '*victim*' biofilm makes AIP as a signaling molecule. AgrC expressed in the '*killer*' detects the AIP and phosphorylates AgrA, which then activates the synthesis of Dispersin B. Dispersin B secreted by the '*killer*' eventually disrupts the '*victim*' biofilm. Figure 8.3 describes this concept.

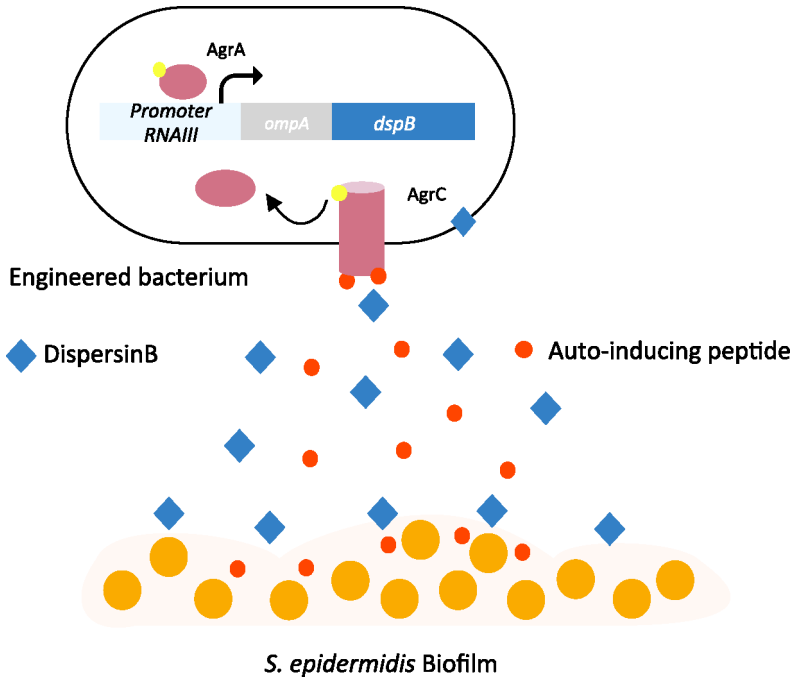

Figure 8.3. – Design of the proposed system exploiting the quorum-sensing system of *S. epidermidis*

9. Dispersin B efficiently disrupts *E. coli* biofilms

Among various anti-biofilm agents explored so far, matrix-degrading enzymes are the most attractive choice. Dispersin B is one of the well-studied matrix-degrading enzymes, which has been demonstrated to be effective on *E. coli* biofilms. This chapter will describe the efficient action of Dispersin B on *E. coli* biofilms and explain why *E. coli* TRMG1655 is the best suitable strain to be regarded as a '*victim*'.

9.1. Cloning and purification of Dispersin B

In order to demonstrate the activity of Dispersin B on *E. coli* biofilms, it was essential to clone, express and purify the enzyme. The plasmid pJK618, was used as a template to amplify the CDS of Dispersin B, which was then cloned into the vector pQE60 along with a 10X Glycine linker between the CDS and a 6X Histidine tag at the C- terminus. The resulting plasmid was named as pVG22, which is illustrated in figure 9.1A.

To find the optimal condition for expression of Dispersin B, *E. coli* M15 cells containing pVG22 were induced at different concentrations of IPTG (isopropyl β-D-1-thiogalactopyranoside). The activity of the enzyme in the cell lysate was measured using a NP-GlcNAc assay (Shibata & Yagi, 1996). This assay involves the use of a substrate 4-Nitrophenyl N-acetyl-β-D-glucosaminide (NP-GlcNAc), which mimics the β, 1⇒6 linkage found in PGA. Upon action of Dispersin B, 4-nitrophenoxide is released as a product, which can be quantified by reading the absorbance of the reaction mixture at 400 nm. The activity of the enzyme was found to be saturating after induction by 100 μM of IPTG (figure 9.1B). It was also observed that cell growth was compromised at concentrations of IPTG higher than 100 μM. I therefore used 100 μM of IPTG for the expression and purification of Dispersin B.

Dispersin B was purified by using Ni-IDA packed columns from Machery-Nagel (figure 9.1C), and the enzyme was sufficiently enriched in the process of purification (figure 9.1D). The amount of protein in the final stage of purification was ~ 6 mg/ml.

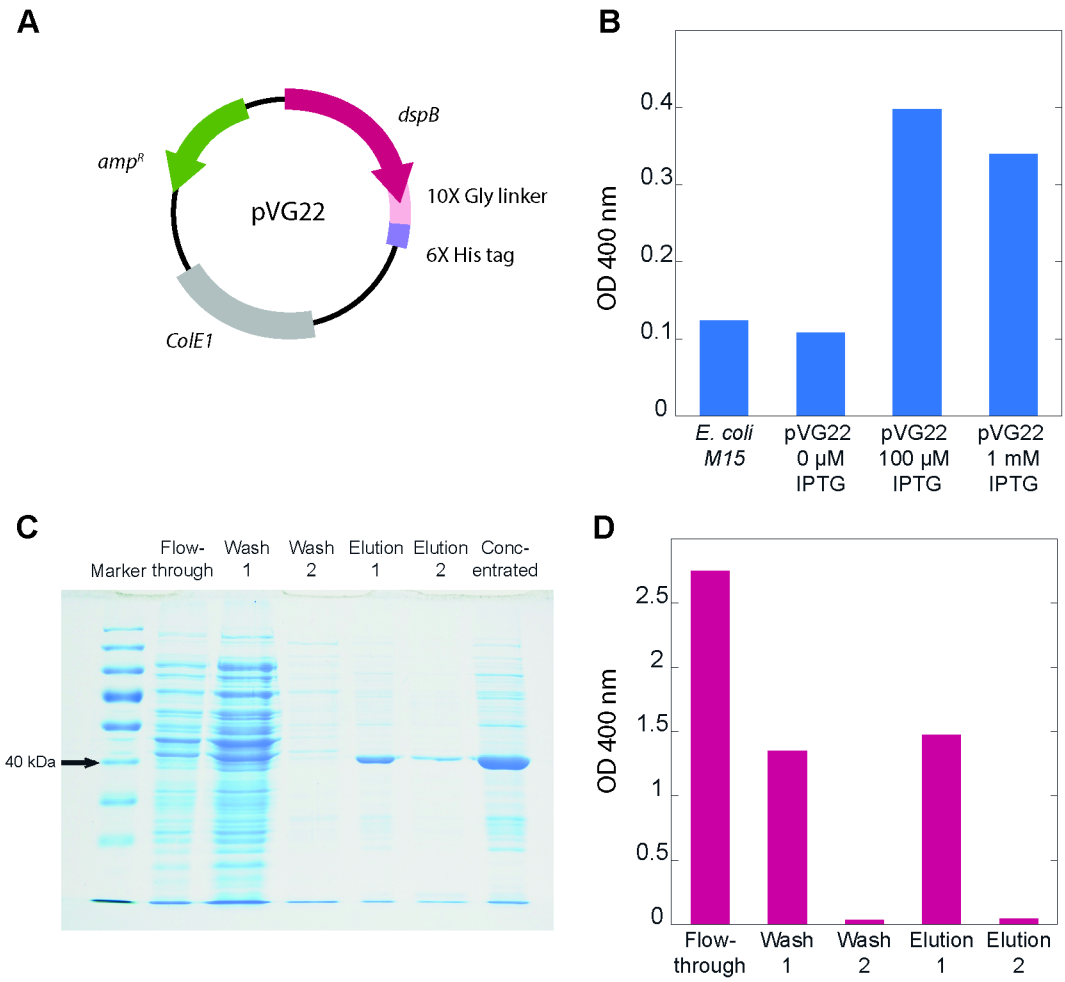

Figure 9.1. – Cloning and purification of Dispersin B **A**. Design of the construct pVG21, **B**. Activity of Dispersin B in the cell lysate upon induction at various concentrations of IPTG, **C**. SDS-PAGE analysis of the different steps of purification of Dispersin B, **D**. Activity of Dispersin B in the different steps of its purification

9.2. The effect of Dispersin B on various strains of *E. coli*

Biofilms of different strains of *E. coli* were grown in 96-well microtiter plates. The 24 h old biofilms were treated with purified Dispersin B for one hour, and the effect of the treatment was quantified by crystal violet staining. This simple method is semi-quantitative, as it measures only cell attachment and does not consider three-dimensional biofilm structure. As described earlier, a biofilm is not just a layer of attached cells but rather a dynamic three-dimensional structure. Though this structure is an important element of biofilms, in this case, the intent was only to check if the biofilm is disrupted by enzyme treatment. Thus, the crystal violet staining method, despite being semi-quantitative, was sufficient for studying biofilm disruption for the purpose of this project.

9.2.1. The effect of Dispersin B on the biofilms of WT strains of *E. coli*

There are various WT strains of *E. coli* used in the scientific community, each of which has a different capacity for biofilm formation and a different biofilm architecture. I have used the strains MG1655, W3110, W3110RH and W3110AR (section 2.2) to check for the effects of Dispersin B. It was observed that there was ~ 20% reduction in biofilm formation of *E. coli* MG1655 due to the treatment with Dispersin B. For other strains, however, there was no significant effect (figure 9.2a). Biofilm formation by the same set of strains was carried out in the presence of Dispersin B. It was found that all strains showed 20-40% reduction in biofilm formation when grown in the presence of Dispersin B (figure 9.2b). This observation confirmed the fact that PGA is more relevant in the initial stage of biofilm formation than in the later stages of biofilm maturation.

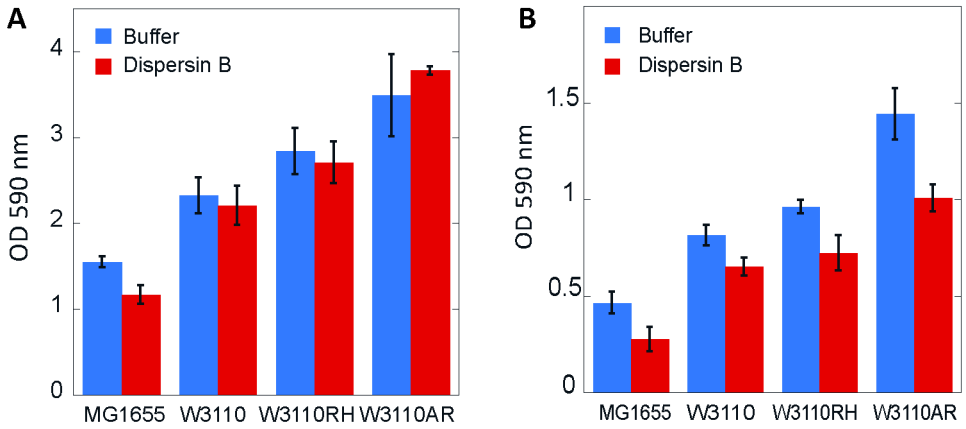

Figure 9.2. – The effect of Dispersin B on various WT strains of *E. coli* **A**. Pre-formed biofilms treated with 60 µg/ml of Dispersin B, **B**. Biofilms grown in the presence of 60 µg/ml of Dispersin B

9.2.2. The effect of Dispersin B on the biofilm of *E. coli* TRMG1655

The protein CsrA regulates the switch from the planktonic state to the biofilm-lifestyle. It drives the cell towards motility and reduces its likelihood of investing in biofilm production. The *csrA* mutant of *E. coli* (TRMG1655, shortly referred as TRMG) exhibits hightened biofilm formation. Since the effect of Dispersin B on this strain was already documented (Itoh *et al.* , 2005), I decided to compare the TRMG strain with WT strains of *E. coli* used above for their susceptibility to Dispersin B. The TRMG strain formed more robust biofilms in comparison to WT under the same set of conditions. The TRMG biofilm was grown for 24 h as well as 48 h, and the effect of Dispersin B on it was assessed by crystal violet staining. Dispersin B treatment disrupted most of 24 h old biofilms and induced

a ~ 40% reduction in 48 h old biofilm biomass (figure 9.3). Almost 100% removal of 24 h old TRMG biofilm was dramatic in comparison to 20% removal of its isogenic WT. This experiment confirmed that TRMG was the best suitable strain to be considered as a '*victim*'.

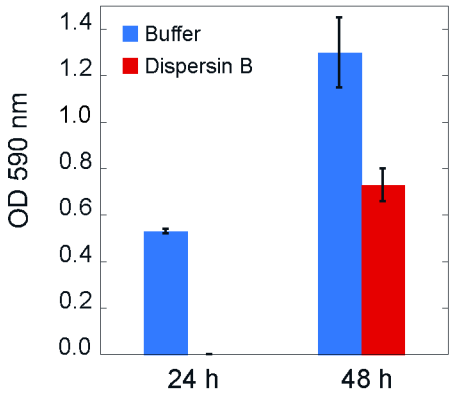

Figure 9.3. – The effect of Dispersin B (60 µg/ml) on the 24 h old pre-formed biofilm of *E. coli* TRMG1655

9.3. Secretion of Dispersin B

The proposed system requires that the anti-biofilm agent Dispersin B should be synthesized and secreted by '*killer*' cells. To make *E. coli* cells secrete Dispersin B, it was essential to choose the right strategy for secretion. I chose the simplest method of fusing the secretion signal of OmpA to the enzyme. The nucleotide sequence encoding first 21 amino acids of OmpA was fused to the CDS of Dispersin B at its 5' end and the resultant fragment was cloned into the vector pTrc99a. The resultant construct was named pVG30 (figure 9.4A). The *E. coli* cells harboring pVG30 were induced with various concentrations of IPTG and grown in the medium containing NP-GlcNAc. After an overnight growth, presence of the enzyme in the medium was confirmed measuring the OD of the spent medium at 400 nm. It was observed that fusing the secretion signal of OmpA to Dispersin B successfully drove it out of the cell (figure 9.4B). The maximum amount of enzyme was found to be secreted when the cells were induced at 100 µM of IPTG.

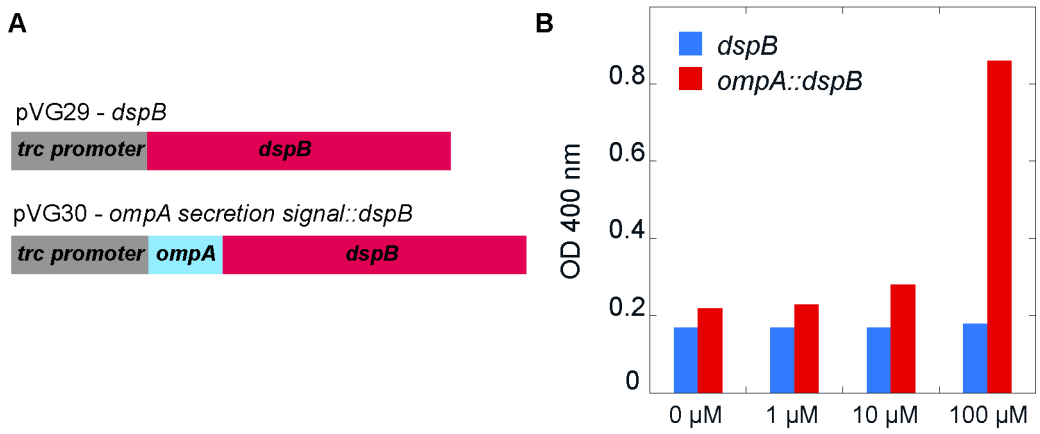

Figure 9.4. – The 'killer' cell secretes Dispersin B **A**. The design of the constructs pVG29 and pVG30, **B**. Secretion of Dispersin B from the cells harboring different constructs induced with different concentrations of IPTG

9.4. The 'Killer' secreting Dispersin B disrupts the '*victim*' biofilm

To demonstrate the effect of the secreted enzyme on the '*victim*' biofilm, a pre-formed biofilm of TRMG was treated with *E. coli* W3110 cells harboring pVG30. The expression of OmpA::DspB was induced by adding 100 µM of IPTG. The effect of this treatment was quantified by crystal violet staining as described previously. The TRMG biofilm was reduced by 87% due to Dispersin B secreted by the '*killer*' (figure 9.5a). Different combinations of the inducer concentration and treatment time were attempted in order to determine the most suitable condition for this treatment. It was evident that a minimum of 12 h of treatment was necessary to observe an approximately 50% reduction in the TRMG biofilm (figure 9.5b). In conclusion, Dispersin B secreted by the '*killer*' cells was able to successfully disrupt the '*victim*' biofilm.

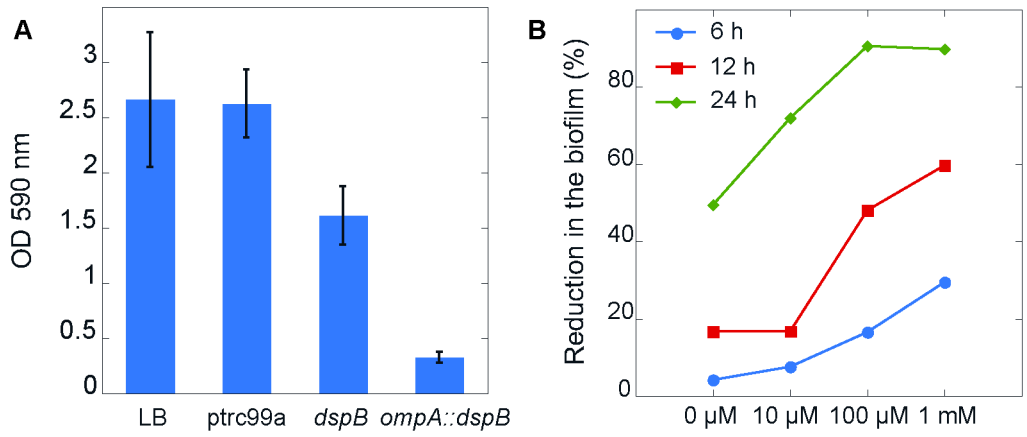

Figure 9.5. – The 'killer' secreting Dispersin B disrupts the biofilm of *E. coli* TRMG1655 **A**. The 24 h old pre-formed biofilm of *E. coli* TRMG1655 treated with *E. coli* W3110 harboring various constructs, **B**. The disruption of the pre-formed biofilm of *E. coli* TRMG1655 by Dispersin B secreted by *E. coli* W3110 harboring the construct pVG30, induced at different concentrations of IPTG for variable time

10. Characterization of the NAG signaling pathway of *E. coli*

After demonstrating the successful action of the '*killer*' on the '*victim*' biofilm, the next step was to combine the expression of Dispersin B with a biofilm-specific signaling pathway. The first strategy is to exploit the NAG signaling pathway of *E. coli*. The '*victim*' biofilm is rich in PGA, a polymer of NAG. In the proposed system, NAG generated from the action of Dispersin B acts as an inducer for activating the expression of Dispersin B. This will generate a system in which, synthesis of Dispersin B continues as long as there is a supply of NAG from the '*victim*' biofilm (figure 8.1). This chapter describes how the NAG signaling pathway was characterized and how it could be combined with the expression of Dispersin B.

10.1. Analysis of the activity of the *nagE* promoter

The first and foremost task in this strategy was to analyze how the signaling and metabolic pathway of NAG responds to extracellular NAG. *E. coli* harbors the *nagE/BACD* operon that contains genes encoding enzymes involved in the metabolism of NAG. The organization of the operon and functions of its constituent genes are well known (Plumbridge, 1991). The intergenic region between *nagE* and *nagB* was cloned into the cloning vector pUA66. This cloning vector contains the CDS of *gfp*mut3 and a set of restriction enzyme sites upstream (Zaslaver *et al.* , 2006). Any putative promoter could be cloned in these sites and its activity studied by measuring GFP expression. The resultant construct was named pVG18 (figure 10.1A). In this construct, the promoter of *nagE* drives the expression of GFP upon induction by NAG.

E. coli W3110 WT cells harboring the construct pVG18 were grown in M9 minimal medium supplemented with varying concentrations of NAG for a few hours, and the level of GFP expressed in them was measured by flow cytometry. Average GFP fluorescence per cell was considered as a proxy for the activity of the *nagE* promoter. Figure 10.1B illustrates GFP fluorescence per cell after three hours of growth in the presence of NAG. The minimum concentration required to activate the *nagE* promoter by two fold was 50 µM.

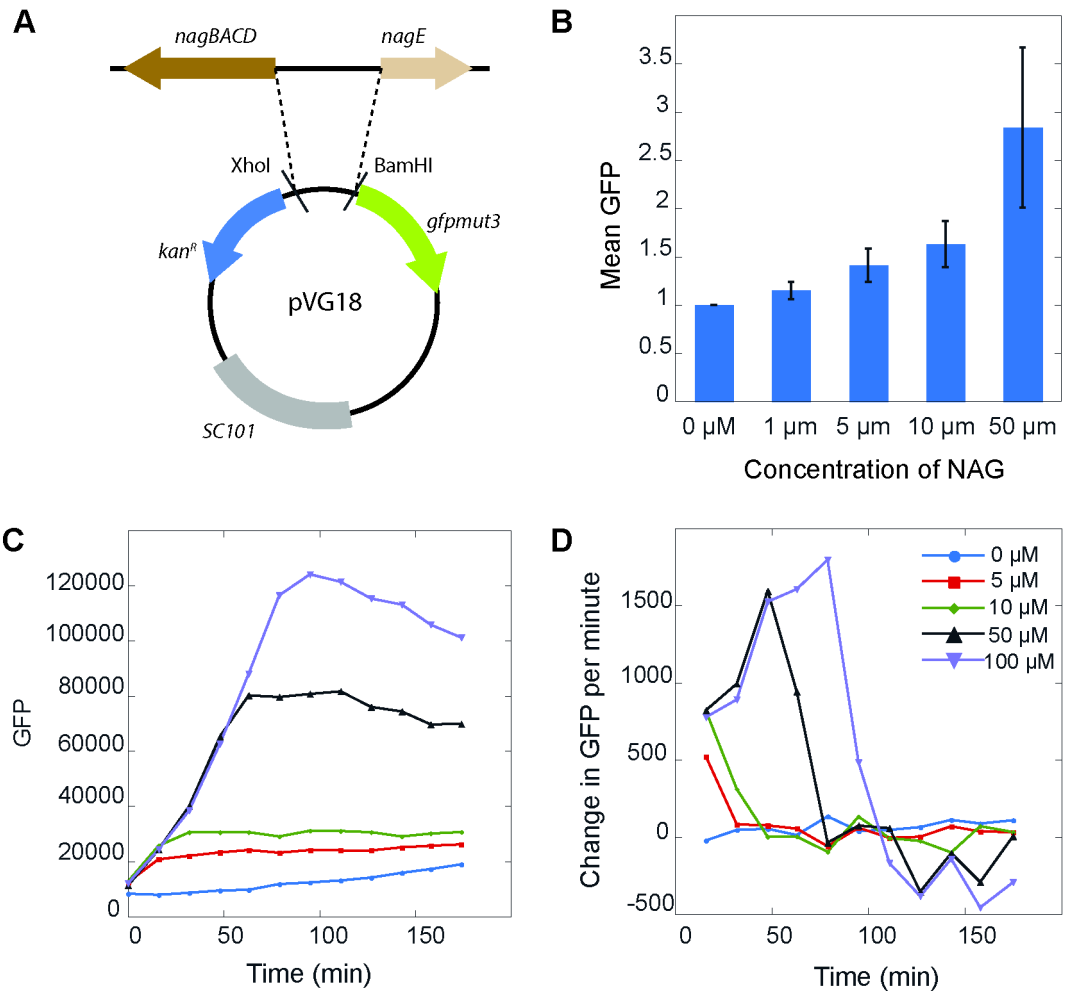

Figure 10.1. – Analysis of the activity of the *nagE* promoter **A**. Design of the construct pVG18, **B**. Activity of the *nagE* promoter at various concentrations of NAG measured by flow cytometry, **C**. Activity of the *nagE* promoter at various intervals of time, **D**. Change in the activity of the *nagE* promoter per unit time

Since cells utilize NAG as a carbon source, the effective concentration of NAG in the medium might have decreased over time. Thus, I decided to measure the expression of GFP at certain intervals of time after induction (figure 10.1C). The change in the expression of GFP per unit time was calculated and normalized to the cell density (figure 10.1D). It was observed that at 5 and 10 µM of NAG, the activity of the *nagE* promoter started declining immediately as cells began to grow and divide. At 50 and 100 µM of NAG *nagE* promoter activity increased for 50 and 80 minutes respectively and declined thereafter. This implied that *E. coli* cells could rapidly utilize small amounts of NAG. The synthesized GFP remained stable inside the cells for the time interval within which the cells were observed. The *nagE* promoter thus responded well to extracellular NAG, however it was not sensitive to low concentrations of NAG.

10.2. Estimating the concentration of NAG in TRMG biofilms

After determining the NAG concentration range within which the *nagE* promoter responds, it was necessary to estimate the concentration of NAG in the TRMG biofilm that is treated with Dispersin B. Biofilms of *E. coli* TRMG contain PGA as the most important constituent. As PGA is a polymer of NAG, it could be expected that TRMG biofilms liberate some NAG due to spontaneous PGA degradation. Treatment with Dispersin B would presumably generate more NAG due to its action on the polymer. Thus, I decided to estimate NAG present in the Dispersin B-treated and untreated biofilm supernatant of TRMG biofilm. I performed MBTH assay (Smith & Gilkerson, 1979) to estimate the concentration of NAG in TRMG biofilm. The reagent 3-methyl-2-benzothiazolone hydrazone (MBTH) reacts with aldehydes in the presence of Fe3+ ions to give rise to a strongly colored complex whose concentration can be measured as the optical density at 650 nm.

TRMG biofilms were grown in 10 ml cultures and supernatants were prepared by scratching the attached cells off the surface (section 7.10). Scratching merely detaches the cells from the surface and does not affect the biochemical nature of matrix components. The supernatant was treated with Dispersin B for one hour and then used for estimation of NAG content. The supernatant of TRMG biofilm contained less than 1 μM of NAG (figure 10.2A). There was a small difference observed between Dispersin B treated and untreated supernatant. These results indicated that there was detectable NAG in the biofilm supernatant. Since PGA is strongly attached to the cell surface (Wang *et al.* , 2004), the procedure of biofilm supernatant preparation might not have isolated entirety of PGA in biofilms. Considering these points, the measurement of NAG in TRMG biofilms could be an underestimate. Regardless of how much NAG was present in TRMG biofilm; it was interesting to see if the *nagE* promoter responded to it.

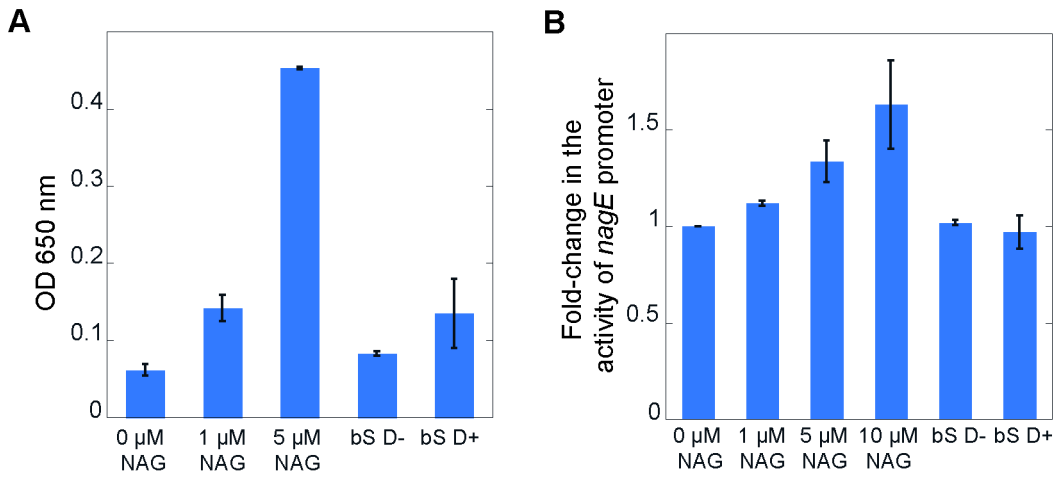

Figure 10.2. – **A**. Estimation of NAG in the biofilm supernatant of TRMG by MBTH assay, **B**. Response of the *nagE* promoter to the biofilm supernatant of TRMG

10.3. Response of the *nagE* promoter to NAG released from the '*victim*' biofilm

Since the *nagE* promoter responded well to the extracellular NAG, it was interesting to check if it could also respond to NAG present in TRMG biofilms. I used the same biofilm supernatant that was subjected to MBTH assay above to check for its effect on the activity of the *nagE* promoter. The cells were grown in the presence of the biofilm supernatant and the activity of the *nagE* promoter was measured by flow cytometry. It was observed that the biofilm supernatant, whether treated with Dispersin B or untreated was unable to activate the *nagE* promoter above its basal level of expression (figure 10.2B). The procedure of biofilm supernatant preparation was the same as that used above for chemical estimation of NAG. It was already stated that this procedure might not have isolated all of PGA found in the biofilm. As a result, this procedure might have diluted NAG to such an extent that its final concentration was too low to induce any change in the activity of the *nagE* promoter.

To circumvent the issue of dilution, it was decided to directly add cells containing pVG18 (*nagE* reporter cells) on the pre-formed TRMG biofilm and test the activity of the *nagE* promoter following the treatment with Dispersin B. When the reporter cells stay in close contact with the TRMG biofilm, NAG released from the TRMG biofilm surrounds them. In that case, reporter cells would be directly exposed to the actual concentration of NAG in the TRMG biofilm and respond to it. In order to test this hypothesis, a pre-formed TRMG biofilm was treated with Dispersin B and reporter cells were added to it. The treatment was performed for one hour. The treated biofilm was scratched and tested for the activity of the *nagE* promoter by flow cytometry. The outcome of the experiment is shown in figure 10.3. The basal expression from the *nagE* promoter was much less than that in the

above experiment. It was difficult to distinguish between the populations of the '*killer*' and the '*victim*'. It was evident from the scatter plots that the treatment with Dispersin B indeed disrupted the large clumps of cells in the TRMG biofilm. Individual cells of the TRMG biofilm formed as a result of this disruption overlapped with the reporter cells. This complicated the interpretation of the effect of Dispersin B treatment on the activity of *nagE* promoter. However, no obvious increase in the activity of the *nagE* promoter was observed.

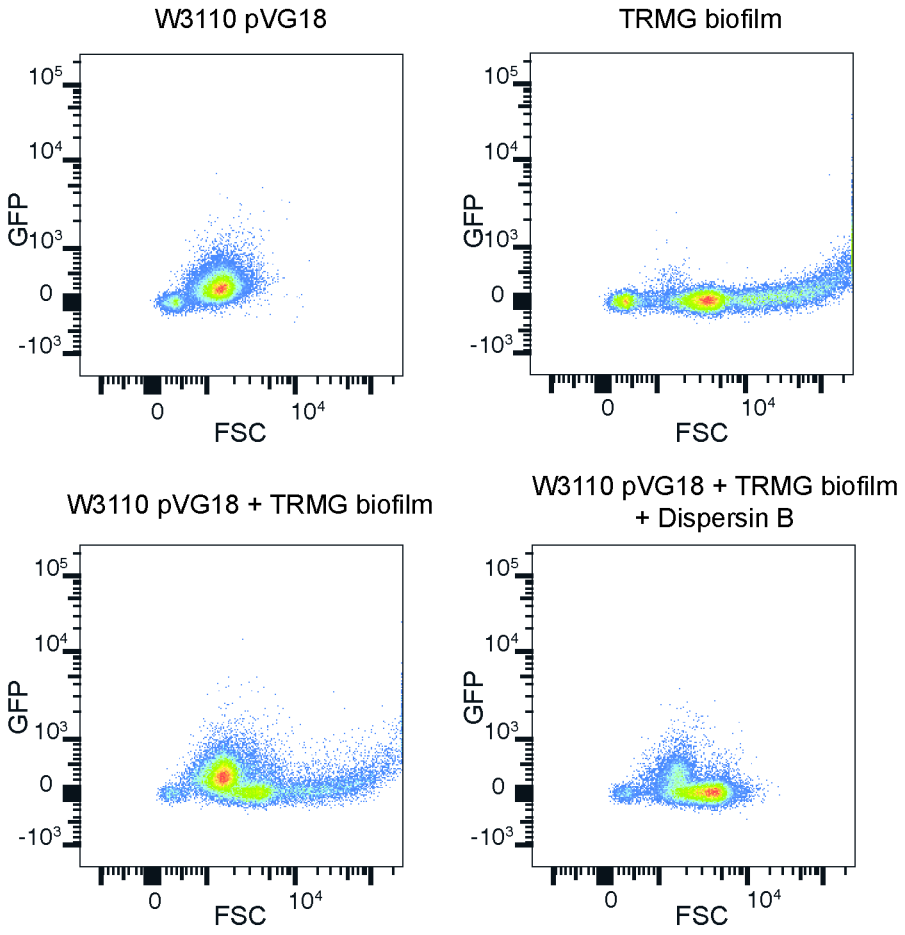

Figure 10.3. – The activity of the *nagE* promoter when the reporter cells were incubated with the Dispersin B-treated TRMG biofilm

10.4. Overexpressing the *pgaABCD* operon in TRMG biofilm

Considering the observations from the previous experiments it was clear that the TRMG biofilm does not make enough PGA so that its degradation generates NAG that will activate the *nagE* promoter. However, the concept of synthesizing Dispersin B from the *nagE* promoter might still work if the biofilm makes more PGA. In order to test this hypothesis, entire *pgaABCD* operon was cloned and expressed in the TRMG strain. Biofilm formation

under different levels of overexpression of *pgaABCD* operon was studied by crystal violet staining. Overexpression of the *pgaABCD* operon at low levels of induction reduced biofilm formation.The highest level of overexpression of the *pgaABCD* operon resulted in less than a 2.5 fold increase in biofilm formation with respect to the TRMG strain (figure 10.4A). Since there are many other factors involved in biofilm formation, higher synthesis of PGA might not result in higher biofilm formation. However, the biofilm may contain the polymer of PGA in excess amount. Thus, I prepared the supernatant from the TRMG biofilm overexpressing the *pgaABCD* operon at the highest possible level and tested it for the activation of the *nagE* promoter. Unfortunately, the *nagE* promoter did not show any increase in its activity above the basal level in the presence of the biofilm supernatant (figure 10.4B). This observation indirectly suggested that overexpressing genes involved in PGA synthesis resulted in higher synthesis of the polymer, however its degradation into NAG was not sufficient to induce the *nagE* promoter.

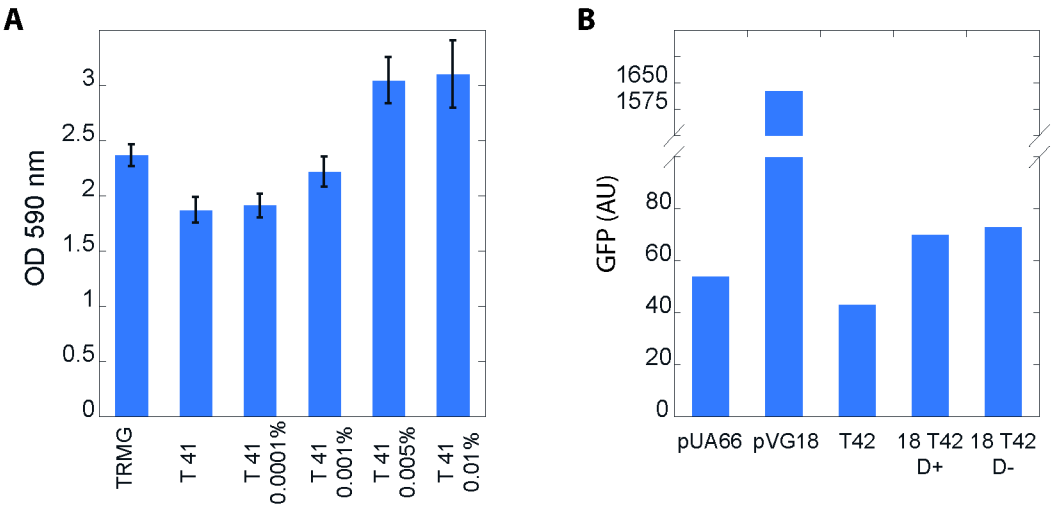

Figure 10.4. – Overexpressing the *pgaABCD* operon in the TRMG biofilm **A**. Biofilm formation at various levels of induction of the *pgaABCD* operon, **B**. Activity of the *nagE* promoter when reporter cells were incubated with the Dispersin B-treated TRMG biofilm overexpressing the *pgaABCD* operon

10.5. Response of the *nagE* promoter to the biofilm supernatant of *S.* epidermidis

The biofilm of *S. epidermidis* contains PIA (polysaccharide intercellular adhesin) as a major component. This polysaccharide is essentially the same as the PGA polymer found in *E. coli*. It was also shown that Dispersin B successfully disrupts *S. epidermidis* biofilms (Kaplan *et al.* , 2004). Thus, I decided to test the activity of the *nagE* promoter in the presence of Dispersin B-treated biofilm supernatant of *S. epidermidis*. The biofilm supernatant was prepared in the same way as that of *E. coli* and was treated with Dispersin

B for one hour. *E. coli* cells harboring the construct pVG18 were grown in the presence of the biofilm supernatant and the activity of the *nagE* promoter was measured by flow cytometry. Unfortunately, there was no significant increase observed in the activity of *nagE* promoter in the presence of the biofilm supernatant (data not shown).

10.6. Improving the sensitivity of the *nagE* promoter

The observations above made it clear that the TRMG biofilm did not make enough PGA to generate NAG that could activate the *nagE* promoter under the culture and assay conditions used here. However, it could be possible to alter the components of the *nagBACD* operon and improve the sensitivity of the *nagE* promoter so that it responds to the concentration of NAG lower than its normal level of activation. Here, sensitivity refers to the fold-change in the activity of the *nagE* promoter at concentrations of NAG between 1 to 10 μM.

Since NAG is a metabolite, there is always some amount of NAG present inside the *E. coli* cell. This NAG is in the form of NAG-6-phosphate, which is able to bind to NagC and de-repress the expression of *nagE* and *nagBACD*. This basal level of NAG-6-P contributes to the activity of the *nagE* promoter without any induction from NAG present in the surrounding medium. In short, the *nagE* promoter has a high level of leaky expression due to the presence of intracellular NAG-6-P. If this intracellular NAG-6-P could be reduced, it would result in low basal activity of the *nagE* promoter and consequently, higher fold-change in its activity around 1 μM of NAG.

10.6.1. Overexpression of NagC

One way to improve the sensitivity of the *nagE* promoter could be overexpression of NagC. High levels of NagC would saturate the existing NAG-6-P and the repressor would be free to respond to NAG being taken up from the environment. To test this hypothesis, the *nagC* gene was cloned and co-expressed along with pVG18 in *E. coli* W3110 cells. The cells were grown in the presence of NAG and the activity of the *nagE* promoter was estimated by flow cytometry. Figure 10.5A shows the activity of the *nagE* promoter at different concentrations of NAG when NagC was overexpressed by adding 100 µM of IPTG. It is clear that the overexpression of NagC reduced the activity of the *nagE* promoter at different concentrations of NAG. The fold-change in *nagE* promoter activity at a particular concentration of NAG decreased slightly until 10 µM of NAG and increased thereafter. However, the fold-change was always less than unity, indicating reduction in *nagE* promoter activity at all concentrations of NAG. Figure 10.5B shows the activity of the *nagE* promoter normalized to its basal expression. It reflects the same trend as that of figure A. The fold-change in the activity of the *nagE* promoter at concentrations of NAG between 1 to 10 µM remained unaffected in the presence of overexpressed NagC. The overexpression of the repressor therefore did not result in the intended improvement in the sensitivity of the *nagE* promoter.

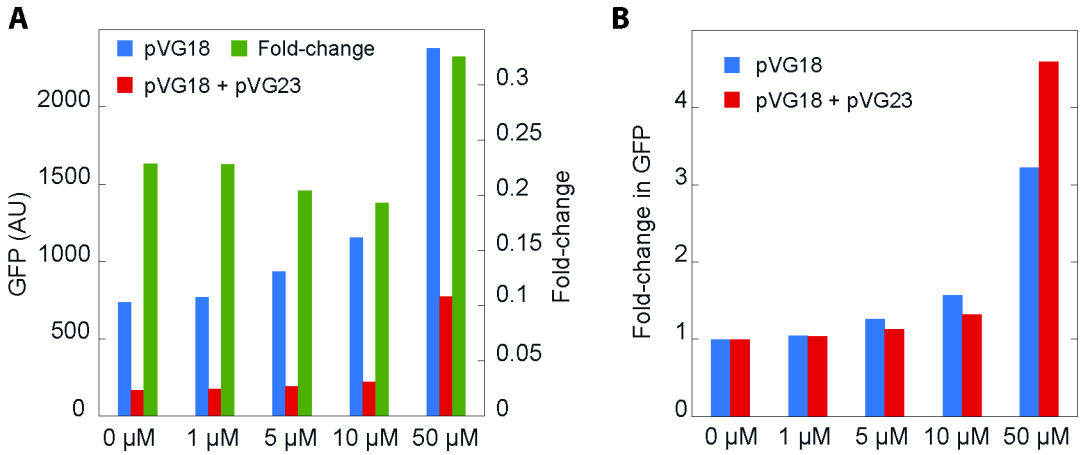

Figure 10.5. – Effect of the overexpression of NagC on the activity of the *nagE* promoter **A**. Activity of the *nagE* promoter at various concentrations of NAG in the presence of NagC and the fold-change in the activity at respective concentrations of NAG, **B**. Activity of the *nagE* promoter normalized to its basal activity at various concentrations of NAG in the presence of overexpressed NagC

10.6.2. Overexpression of NagB and NagA

Another way to reduce the basal activity of the *nagE* promoter would be to overexpress the enzymes involved in the catabolism of NAG. High levels of NagB and NagA would catabolize the existing NAG-6-P and keep the repressor bound to the operator region of the *nagE* promoter. This would eventually reduce the basal activity of *nagE* promoter. To test this hypothesis, *nagB* and *nagA* genes were cloned in a single bicistronic construct and co-expressed along with pVG18, and the activity of the *nagE* promoter was measured in the same manner as above. Figure 10.6A shows the activity of the *nagE* promoter at different concentrations of NAG. The fold-change in the activity at a particular concentration of NAG descreased continuously with the increasing concentration of NAG. However, like the overexpression of NagC the fold-change was always less than unity, implying the reduction in *nagE* promoter activity. Figure 10.6B shows the activity of the *nagE* promoter normalized to its basal expression. The fold-change at concentrations of NAG between 1 to 10 µM was lowered in the presence of overexpressed NagB and NagA. In conclusion, this modification also could not result in improving the sensitivity of the *nagE* promoter.

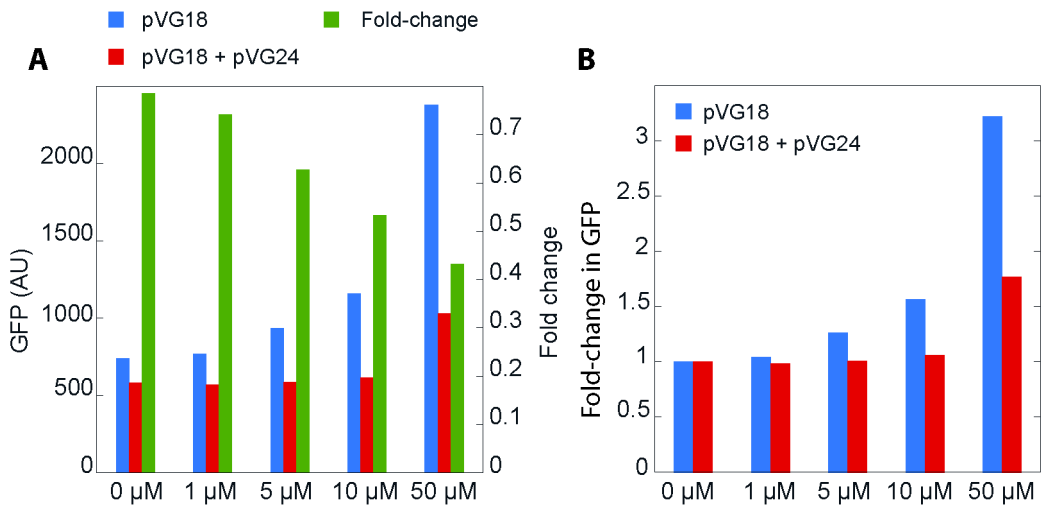

Figure 10.6. – Effect of the overexpression of NagB and NagA on the activity of the *nagE* promoter **A**. Activity of the *nagE* promoter at various concentrations of NAG in the presence of overexpressed NagB and NagA and the fold-change in the activity at respective concentrations of NAG, **B**. Activity of the *nagE* promoter normalized to its basal activity at various concentrations of NAG in the presence of overexpressed NagB and NagA

10.6.3. Overexpression of NagE

The last possibility of achieving the improved sensitivity of the *nagE* promoter was to overexpress NagE- the transporter of NAG. The greater the number of transporters, the greater the amount of NAG taken up by the cell. This may enhance the ability of the cell to take up NAG from the environment even if its concentration in the surrounding medium is low. It would eventually lead to higher activity of the *nagE* promoter at low extracellular concentration of NAG. To test this hypothesis, the *nagE* gene of *E. coli* was cloned and co-expressed with pVG18, and the activity of the *nagE* promoter was measured. In this case, the change in the activity of the *nagE* promoter was monitored every 15 minutes while growing the cells simultaneously inside the Tekan microplate reader. There was no change observed in the activity of the *nagE* promoter due to the overexpression of *nagE* (data not shown). The last possibility of achieving improved sensitivity of the *nagE* promoter did not materialize.

11. Chemotaxis towards NAG

Chemotaxis is the migration of an organism towards a chemical stimulus. Chemotaxis in *E. coli* is a well-studied signaling pathway. Being relatively simple and robust, it is amenable to manipulation. In the proposed system, exploiting the chemotactic signaling pathway that induces migration towards NAG could enhance efficiency of the system. The '*killer*' would not only move towards the '*victim*' biofilm, but also make Dispersin B in the close vicinity of the '*victim*'. Thus, combining Dispersin B expression with chemotaxis was an attractive strategy. This chapter will describe the characterization of chemotaxis towards NAG and attempts made to improve the chemotactic response of the cells in order to be suitable for the proposed system.

For *E. coli*, NAG is a strong chemoattractant. The first reference to NAG as a chemoattractant for *E. coli* dates to the preliminary work on bacterial chemotaxis by James Adler (Adler, 1976). In this work, chemotaxis of *E. coli* towards NAG was demonstrated by a capillary assay. The methods of analyzing bacterial chemotaxis have evolved over time, and many more compounds are now characterized as chemoattractants. However, there are no further references in the literature concerning chemotaxis towards NAG. Thus, I thought of first characterizing chemotaxis towards NAG with the help of methods available in the lab.

11.1. Analyzing chemotaxis towards NAG by stimulus dependent flow-FRET

With the help of advanced biophysical techniques, it is now possible to quantitatively analyze the signal processing of the chemotactic pathway of *E. coli* (Sourjik *et al.* , 2007). I used the flow-FRET (fluorescence resonance energy transfer) setup in the lab to analyze chemotaxis of *E. coli* towards NAG. Figure 11.1 describes the concept underlying this technique. The proteins CheY and CheZ are fused with YFP and CFP, respectively. When these two proteins interact, FRET takes place and there is a drop in the fluorescence of CFP and gain in that of YFP. In the absence of an attractant CheY and CheZ bind and dissociate as a steady state. Thus, the ratio of YFP and CFP fluorescence remains constant. When an attractant binds to the receptor, the kinase activity of CheA is inhibited, resulting in a sudden drop of CheY-P. Subsequently, CheY and CheZ dissociate, which is followed by

a gain in the fluorescence of CFP and drop in that of YFP. The change in the kinase activity of CheA can be quantified by monitoring the ratio of YFP and CFP fluorescence. A drop in this ratio indicates the presence of an attractant and a gain in the ratio indicates removal of an attractant.

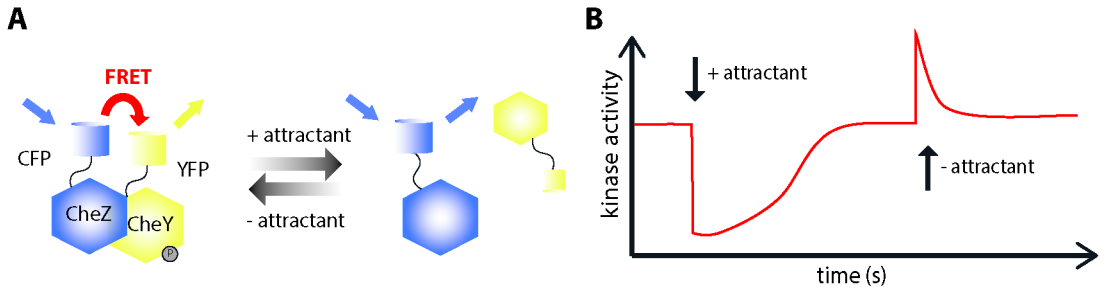

Figure 11.1. – The concept of stimulus-dependent flow-FRET **A**. Interaction of CheY-YFP and CheZ-CFP in the presence or absence of the attractant, **B**. Change in the kinase activity in the presence or absence of the attractant

To analyze the chemotactic response of *E. coli* towards NAG, buffer-adapted cells were stimulated with various concentration of NAG and the kinase activity of CheA in response to the stimulus was measured. It was found that NAG was able to inhibit the kinase activity in the range of 1 to 10 µM (figure 11.2).

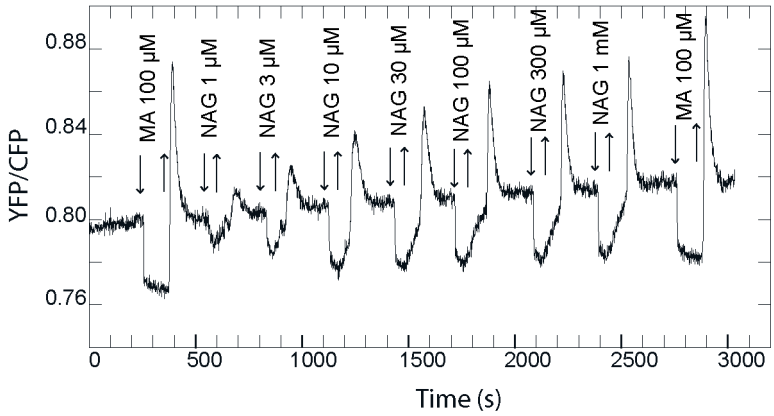

Figure 11.2. – Chemotaxis of *E. coli* LJ110 towards NAG analyzed by stimulus-dependent flow-FRET

As evident from the above experiments, biofilm of TRMG contains not more than 10 µM of NAG. The concentration of NAG, which is already low in the biofilm, is likely to drop several fold when it diffuses away from the biofilm. For chemotaxis towards NAG to be helpful for the proposed system, the '*killer*' must be able to respond to NAG at some distance away from the biofilm. Thus, the system would work only if the '*killer*' shows chemotaxis towards NAG at concentrations within nanomolar range. In order to design a strategy for improving chemotactic response towards NAG, it was important to understand the exact mechanism of chemotaxis towards NAG.

11.2. Chemotaxis towards NAG is PTS-mediated and not receptor-mediated

As described earlier, *E. coli* shows chemotaxis to various compounds via either receptor (MCP) mediated pathway or PTS-mediated pathway. Generally, chemotaxis towards sugars occurs via PTS-mediated pathway. Since NAG is a sugar, chemotactic response to it was more likely to occur via PTS, however, the possibility of chemotaxis through a receptor like Trg could not be ruled out. Thus, I decided to examine the chemotaxis of *E. coli* towards NAG by swarm plate assay (Wolfe & Berg, 1989). In this assay, an attractant is spotted in the center of a Petri plate containing M9 minimal medium with 0.225 % agar. In soft agar, cells can swim through the pores created by the polysaccharide network. As they spread from the point of inoculation, they consume nutrients and thus create a nutrient gradient. This results in the formation of a ring with distinct alternating regions of cell density. When cells move more towards an attractant, they make an egg-shaped ring pointing towards the increasing gradient of the attractant.

In this experiment, the goal is to unravel the exact pathway of chemotaxis towards NAG. Thus, chemotaxis of the WT *E. coli* LJ110 was tested along with some suitable mutants. The first and foremost mutant that was constructed was *nagE* deletion mutant (*ΔnagE*). The second mutant was a double deletion mutant of *ptsI* and *cpdA* (*ΔptsIΔcpdA*). This deletion abolishes all the phosphotransferase systems in the cell. The third mutant used was a strain of *E. coli*, in which all five MCPs are deleted (UU1250). It has no functional cluster of MCP-CheA-CheW and it does not show any chemotactic response towards any compound. The attractant, which is 100 mM of NAG was spotted at the center of the soft-agar plate and allowed to diffuse in the agar overnight. Overnight cultures of the above mentioned mutants were spotted at positions equidistant from the center. The plate was incubated to allow the formation of the swarm rings. Figure 11.3 shows the results of the experiment. The WT of *E. coli* showed a conspicuous egg-shaped swarm ring pointing towards the increasing concentration of NAG. The Δ*nagE* strain formed a much smaller swarm ring in comparison to the WT strain. This could be due to slower growth rate of Δ*nagE* strain on minimal medium. It showed chemotaxis towards methyl aspartate, which implied that it was not impaired in the chemotactic signaling pathway. It did not show any chemotaxis towards NAG. The strain UU1250 showed no chemotaxis towards methyl aspartate or NAG. Its phenotype on the plate was an example of complete lack of chemotaxis (negative control). The *ΔptsIΔcpdA* strain showed a chemotactic response towards methyl aspartate, implying its functional chemotactic signaling pathway. However, it showed no chemotaxis towards NAG, which conveyed that PtsI plays an essential role in chemotaxis towards NAG.

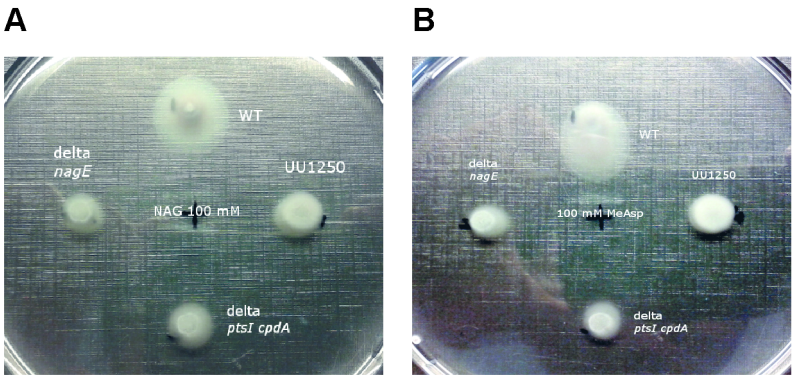

Figure 11.3. – Swarm plate assay for chemotaxis **A**. towards 100 mM NAG, **B**. towards 100 mM methyl aspartate

Although the swarm plate assay implied that chemotaxis towards NAG is PTS-mediated, it was not reliable due to different growth rates of mutants on the minimal medium. Thus, I decided to test these mutants by stimulus dependent flow-FRET for chemotaxis towards NAG. The deletion mutant of *nagE* showed the same response to NAG as that of the WT strain. Surprisingly, deleting the transporter of NAG had no effect on the concentration range within which the cells showed inhibition of kinase activity (figure 11.4A). The amplitude of the drop in the ratio of YFP and CFP signal also remained the same, as that of the WT strain. In short, other transporters like ManXYZ, which also transport NAG inside the cell, were able to compensate for the lack of NagE. The double knockout of *ptsI* and *cpdA* showed no response to NAG (figure 11.4B). It showed a minute response to glucose, to which chemotaxis is mostly PTS-mediated, but is also known to take place through the receptor Trg. The mutant responded to methyl aspartate in the same manner as that of the WT strain. Chemotaxis to methyl aspartate is entirely receptor-mediated, indicating the presence of a functional chemotactic signaling pathway in the mutant. From these observations, it was concluded that chemotaxis towards NAG occurs entirely through PTS and not through any chemoreceptor.

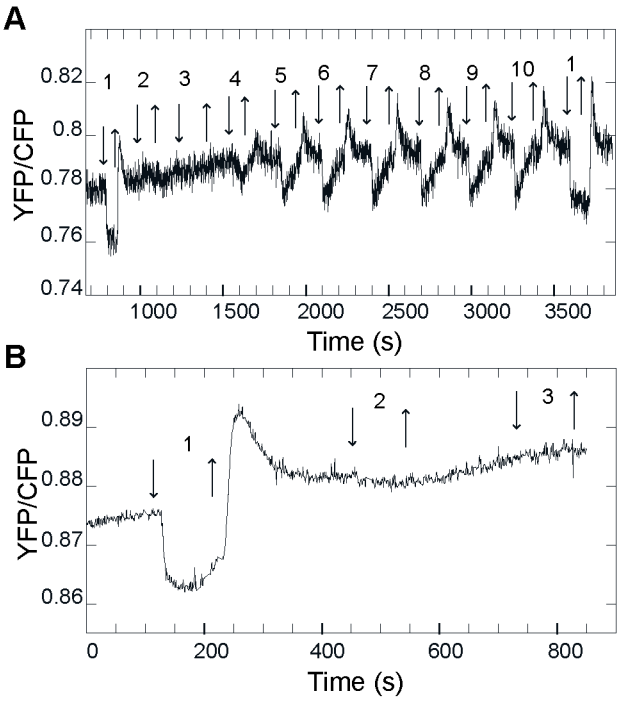

Figure 11.4. – Chemotaxis of the deletion mutants of *E. coli* LJ110 towards NAG analyzed by stimulus dependent flow-FRET **A**. Δ*nagE* (order of the stimuli 1- 100 µM MeAsp, 2- 100 nM NAG, 3- 1 µM NAG, 4- 3 µM NAG, 5- 10 µM NAG, 6- 30 µM NAG. 7- 100 µM NAG, 8- 300 µM NAG, 9- 1 mM NAG, 10- 3 mM NAG), **B**. ΔptsIΔcpdA (order of the stimuli 1- 100 µM MeAsp, 2- 100 µM Glucose, 3- 10 µM NAG)

11.3. Improving chemotaxis towards NAG

Since the chemotactic response of the WT *E. coli* to NAG was not suitable for the proposed system, I considered whether the chemotactic response could be improved. Here, improvement in chemotaxis means enabling the cells to show stronger chemotaxis towards NAG at concentrations lower than 1 µM. The transporter NagE is the chief player involved in the chemotaxis towards NAG. The construct expressing NagE was co-transformed with the construct expressing the FRET-pair in the WT *E. coli*. The transporter NagE was overexpressed by adding different concentrations of the inducer and the chemotactic response towards NAG was measured. Unfortunately, the cells overexpressing the transporter could not respond to NAG under the setup of flow-FRET. This was perhaps due to excess stress caused to the cell due to the overexpression of a membrane protein.

The *ΔnagE* strain had no effect on the chemotaxis towards NAG. Overexpressing the transporter in the *ΔnagE* strain may not cause too much stress to the cell. Thus, I decided to overexpress NagE in the deletion mutant of *nagE* and test the cells for the chemotaxis towards NAG. In this experiment, cells were stimulated with increasing concentrations of NAG without adding the buffer in-between. This method helps us to elucidate the range of the concentration of the attractant within which the cells are responsive to the attractant in spite of adaptation mechanism performed by CheB and CheR (section 4.2.2).

Unfortunately, it was observed that overexpressing *nagE* had no effect on the chemotactic response of the cells towards NAG (figure 11.5).

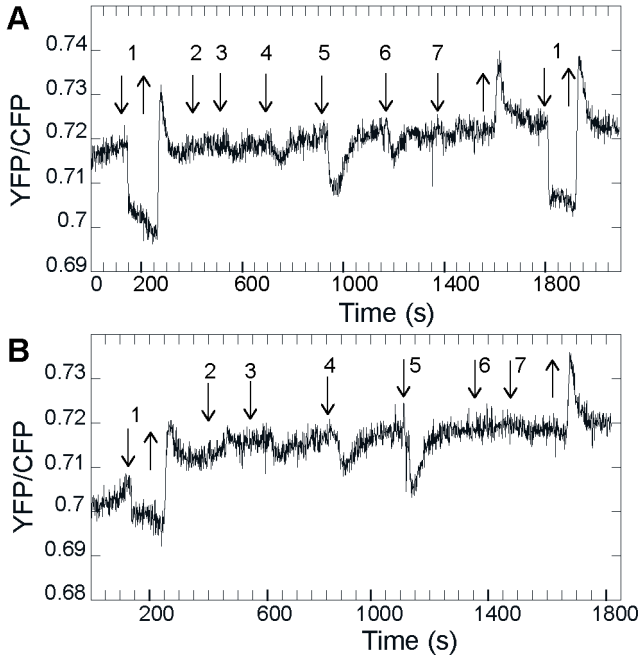

Figure 11.5. – Chemotaxis towards NAG upon overexpression of NagE in the *nagE* deletion mutant of *E. coli* LJ110 **A**. Cells uninduced (order of the stimuli 1- 100 µM MeAsp, 2- 100 nM NAG, 3- 1 µM NAG, 4- 3 µM NAG, 5- 10 µM NAG, 6- 30 µM, 7- 100 µM) **B**. Cells induced with 0.0001% arabinose (order of the stimuli is the same as **A**)

Above experiments made it clear that *E. coli* was naturally chemotactic towards NAG. However, the range of concentration of NAG within which it showed chemotaxis was not sufficient in the context of the proposed system. Attempts made to improve the chemotactic response of *E. coli* also did not succeed. In conclusion, the idea that chemotaxis towards NAG could enhance the efficiency of the proposed system was not feasible.

12. Exploring differential regulation of gene expression in biofilms

The biofilm lifestyle is fundamentally different from the planktonic lifestyle. The coordinated change in the phenotype of bacterial cells during biofilm formation gave rise to the assumption that this change is brought about by a specific pattern of gene expression. Any single gene that is a part of this pattern is likely to get activated by several fold during biofilm formation. That hypothetical biofilm-induced gene is particularly attractive for the proposed system. The promoter associated with this gene can drive the expression of Dispersin B. In this system, Dispersin B would be expressed and secreted as soon as the '*killer*' approaches the '*victim*' biofilm and acquires the biofilm lifestyle. The '*killer*' cells, now a part of the biofilm, would then disrupt the '*victim*' biofilm from within.

Researchers have explored the biofilm-specific gene expression by performing DNA microarray-based identification of genes expressed in *E. coli* biofilm. Some of the key findings of the experiments performed previously have been described (1.5). The most important conclusion of the studies to date is variability in gene expression pattern due to differences in the makeup of the strain, and the conditions used to grow biofilms. In order to find a gene(s) that is up-regulated in the biofilm grown under conditions used in this project, it was essential to sequence its transcriptome.

Genome-wide studies of bacterial gene expression are shifting from hybridization-based technology to next generation sequencing technology. RNA-seq has a number of advantages over using DNA microarrays. It offers annotation-independent detection of transcription, improved sensitivity and increased dynamic range (Croucher & Thomson, 2010). Thus, I decided to perform RNA-seq to explore the pattern of biofilm-specific gene expression. The biofilms of *E. coli* W3110 were grown for 24 hours and planktonic and attached cells were harvested. Exponentially growing cells of the same strain were also harvested. Since the deletion mutant of *fliC* shows significantly less biofilm formation, it was grown in the same conditions as that of the WT biofilms and the planktonic cells (assuming no attachment) were harvested. All these samples were then subjected to the isolation of the total RNA, which was submitted to the sequencing facility.

12.1. Analyzing the data obtained from RNA-seq of *E. coli* biofilms

As the first step of the analysis, the transcriptomes of the attached cells and planktonic cells of the WT biofilms were each compared with that of the exponential phase cells of the WT strain. It was observed that many genes that were up regulated in attached cells with respect to the exponential phase cells were also up regulated in planktonic cells (Figure 12.1). Most of the genes up regulated in biofilms encode proteins involved in metabolic processes such as glycolate metabolism, fatty acid oxidation, oxidoreductase activity, and others. There are also some genes, which are involved in responding to extracytoplasmic stress (*cpxP*), regulation of capsule synthesis (*rcsB*), regulation of curli assembly (*csgD*) and some other regulatory functions. These observations are in agreement with the findings of the previous work (Beloin *et al.* , 2004).

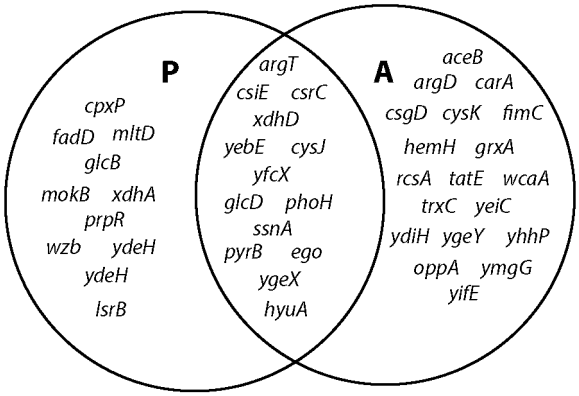

Figure 12.1. – Genes up regulated in planktonic cells and attached cells of the biofilm w.r.t. exponential phase cells of *E. coli* W3110 ($>$ 4 fold, $p < 0.01$)

The transcriptome of the planktonic cells of the deletion mutant of *fliC* was compared with that of the other samples. It was found that the pattern of gene expression in this mutant was similar to that of the planktonic cells of the WT. This implies that the mutant that was unable to form biofilms, was also not showing any similarity in its pattern of gene expression to that of the attached cells of the WT (data not shown).

In order to find the genes that were specifically up regulated in attached cells, the transcriptome of the attached cells was compared with that of the exponential phase cells and the genes that were up-regulated in planktonic cells were filtered out. The result of this filtering is listed in table 12.1 . The interesting observation here is that most of the genes in this list encode enzymes required for oxidative stress. In deeper layers of the biofilm, oxygen concentration drops rapidly. Finding the enzymes involved in combating oxidative stress in the highly anaerobic microenvironment is counter-intuitive. It is interesting to explore into this further.

Table 12.1. – Genes up regulated in attached cells but not in planktonic cells of the biofilm (> 2-fold, $p < 0.05$)

Name	DB Object Name	Attached cells (total RPKM)	Planktonic cells (total RPKM)	Exponential phase cells (total RPKM)	Fold change att vs exp	Fold change pla vs exp
grxA	reduced glutaredoxin 1	12.07	5.38	6.00	67.42	0.65
katG	catalase/peroxidase HPI	11.89	6.81	7.15	26.67	0.79
oxyS	Putative hydrogen peroxide-inducible genes activator	7.33	1.77	2.81	22.86	0.48
hemH	ferrochelatase	9.13	5.44	5.58	11.75	0.91
ahpF	alkyl hydroperoxide reductase, F subunit	11.26	6.41	7.72	11.58	0.40
yaaA	protein that reduces intracellular iron levels under peroxide stress	9.37	5.51	5.89	11.12	0.77
sufA	iron-sulfur cluster assembly scaffold SufA	8.71	3.41	5.73	7.90	0.20
ycaD	YcaD MFS transporter	7.14	4.01	4.24	7.44	0.85
yifE		12.12	9.11	9.38	6.70	0.83
ahpC	alkyl hydroperoxide reductase, C subunit	13.29	10.00	10.66	6.18	0.63
ymgG		4.39	1.66	2.11	4.86	0.73
xthA	exodeoxyribonuclease III	9.07	6.47	7.12	3.88	0.64
yfiP		7.08	4.84	5.36	3.28	0.70

yfhD	membrane-bound lytic murein transglycosylase F	6.40	4.35	4.69	3.28	0.79
speC	Ornithine decarboxylase SpeC	6.65	4.77	5.08	2.97	0.81
ykgJ	predicted ferredoxin	4.19	2.64	2.67	2.87	0.98
trxB	thioredoxin-disulfide reductase	9.43	6.90	8.01	2.67	0.46
uraA	uracil permease	6.67	4.27	5.34	2.53	0.48

12.2. Analyzing protein synthesis of biofilm-induced genes

A few genes that were up regulated only in attached cells and not in planktonic cells were selected. These selected genes are highlighted in the table x. The nucleotide sequences lying upstream to these genes are likely to contain the regulatory elements that regulate their expression. These nucleotide sequences, henceforth called promoters were cloned in the vector pUA66 to generate reporter constructs. In the reporter construct, expression of GFP acts as a proxy for the expression of the gene. Cells harboring these reporter constructs were grown in the same way as they were grown for RNA isolation and the level of GFP expressed in them was measured using flow cytometry. Unfortunately, out of the tested constructs, only pVG56 (*pahpC::gfp*) showed detectable expression. However, the level of expression from this promoter was found to be the same in all growth phases (data not shown).

Planktonic cells in a biofilm represent those cells that are detached from the surface or not yet attached to the surface. These cells remain in the close proximity of the biofilm in the given settings of biofilm cultivation. Thus, genes up regulated in planktonic cells can also be exploited to drive the synthesis of Dispersin B. In order to select such genes, the transcriptome of planktonic cells was compared to that of the exponential phase cells (table 12.2) and a few genes were shortlisted. Selected genes are highlighted in table 12.2. The genes *rmf*, *aceB, astC*, *rpoS* and *fim* were also included in the selected genes that showed up regulation at $p < 0.05$ (data not shown).

Table 12.2. – List of the genes up regulated in planktonic cells w.r.t. exponential phase cells (> 4-fold)

Name	DB Object Name	Planktonic cells (total RPKM)	Exponential phase cells (total RPKM)	Fold change	P value	T value
glcD	glycolate oxidase, subunit GlcD	8.12	1.60	92.00	0.01	12.81
ssnA	predicted chlorohydrolase/aminohydrolase	6.84	1.28	47.13	0.01	17.80
cpxP	Periplasmic stress adaptor protein CpxP	12.00	6.82	36.47	0.01	20.29
fadE	acyl-coenzyme A dehydrogenase	8.64	3.61	32.76	0.01	22.66
cysD	sulfate adenylyltransferase, small subunit	8.11	3.14	31.50	0.01	14.34
phoH	ATP-binding protein	9.34	4.45	29.59	0.00	39.65
cysA	sulfate ABC transporter, ATP-binding protein	8.01	3.31	25.95	0.01	20.62
cysU	sulfate / thiosulfate ABC transporter - membrane subunit	7.76	3.13	24.76	0.01	14.58
lsrB	periplasmic AI-2 binding protein LsrB	5.45	0.92	23.11	0.01	13.31
cysW	sulfate ABC transporter, permease protein	7.69	3.20	22.46	0.01	14.41

Table 12.2. – List of the genes up regulated in planktonic cells w.r.t. exponential phase cells (> 4-fold)

Name	DB Object Name	Planktonic cells (total RPKM)	Exponential phase cells (total RPKM)	Fold change	P value	T value
cysJ	sulfite reductase (NADPH) flavoprotein alpha-component	7.77	3.35	21.42	0.01	13.53
ego	AI-2 ABC transporter - ATP binding subunit	5.95	1.57	20.79	0.01	18.15
ygfU	urate:H+ symporter	4.26	-0.06	19.99	0.01	18.67
ygeX	2,3-diaminopropionate ammonia-lyase	7.03	2.76	19.30	0.01	20.48
glcG	glcG protein	8.92	4.74	18.15	0.01	18.84
ygfM	predicted oxidoreductase	6.74	2.73	16.12	0.01	24.11
glcB	malate synthase G	8.77	4.94	14.21	0.00	37.11
xdhD	fused predicted xanthine/hypoxanthine oxidase	6.84	3.09	13.47	0.00	32.58
csrC	a regulatory RNA	14.70	11.02	12.80	0.01	13.06
yghK	glycolate / lactate:H+ symporter	5.77	2.12	12.56	0.01	12.54
xdhA	xanthine dehydrogenase, small subunit	6.33	2.95	10.38	0.00	38.96
yeeD	conserved protein	8.99	5.67	10.00	0.01	12.40
hyuA	hydantoin utilization protein A	5.17	1.93	9.42	0.01	16.37

Table 12.2. – List of the genes up regulated in planktonic cells w.r.t. exponential phase cells (> 4-fold)

Name	DB Object Name	Planktonic cells (total RPKM)	Exponential phase cells (total RPKM)	Fold change	P value	T value
fadD	long-chain-fatty-acid–CoA ligase	9.44	6.23	9.25	0.00	35.08
mokB		9.74	6.58	8.95	0.01	16.50
xdhB	xanthine dehydrogenase, molybdopterin binding subunit	5.27	2.20	8.38	0.01	16.46
ydeH	diguanylate cyclase	7.04	4.25	6.91	0.01	16.89
csiE	stationary phase inducible protein	7.87	5.22	6.27	0.01	14.95
yfcX	fatty oxidation complex, alpha subunit	8.34	5.70	6.26	0.01	20.24
yebE	conserved inner membrane protein	7.41	4.83	5.98	0.01	16.50
wzb	protein-tyrosine phosphatase	2.22	-0.34	5.92	0.01	13.77
pyrB	aspartate carbamoyltransferase	6.28	3.73	5.83	0.01	17.68
argT	lysine / arginine / ornithine ABC transporter - periplasmic binding protein	9.03	6.54	5.59	0.01	13.02
mltD	membrane-bound lytic murein transglycosylase D	9.00	6.79	4.64	0.00	36.41
yobB	conserved protein	6.82	4.66	4.46	0.01	19.97

Table 12.2. – List of the genes up regulated in planktonic cells w.r.t. exponential phase cells (> 4-fold)

Name	DB Object Name	Planktonic cells (total RPKM)	Exponential phase cells (total RPKM)	Fold change	P value	T value
prpR	PrpR DNA-binding transcriptional dual regulator	3.67	1.51	4.45	0.01	15.45
psiF		6.10	3.95	4.43	0.01	16.71
ydcJ		4.67	2.61	4.17	0.01	16.46

Promoters associated with these genes were cloned in the vector pUA66 and the level of expression of GFP was measured in the same way as above. Some of the constructs in this case also did not express any detectable GFP. However, the promoters of *rmf*, *csrB* and *fim* showed high activity in the planktonic cells of the biofilm (figure 12.2). The promoters of the genes *aceB* and *fad* showed more activity in the attached cells than in the planktonic cells. However, their activity was too low to drive the synthesis of Dispersin B.

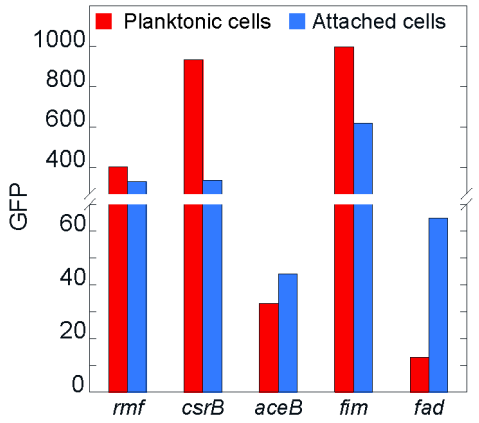

Figure 12.2. – Activity of the selected promoters in the planktonic and the attached cells of the *E. coli* W3110 biofilm

12.3. Expressing Dispersin B from the promoter of the gene *rmf*

The reporter construct of the gene *rmf* was one of those, which showed high activity in the planktonic cells of the biofilm. To test whether the promoter of *rmf* can drive

the synthesis of Dispersin B, it was cloned upstream to *ompA::dspB* construct in the plasmid pVG30. A strong ribosome binding site was introduced before the start codon of *ompA::dspB* (Salis *et al.* , 2009b). The resultant construct was named as pVG46 (figure 12.3A). The cells harboring this construct were tested for the expression and secretion of Dispersin B with the help of the secretion assay as described above. The cells could express and secrete Dispersin B without adding any inducer. The secretion of Dispersin B was almost 50% of that from original *ompA::dspB* construct induced at 100 µM of IPTG (figure 12.3B).

To test the effect of this construct on biofilms, the pre-formed biofilm of *E. coli* TRMG1655 was treated with *E. coli* W3110 harboring the construct pVG46. The cells were able to disrupt the '*victim*' biofilm effectively (figure 12.3). However, the effect of the control construct lacking the OmpA secretion signal was observed to be comparable to that of pVG46. The same control construct did not show any secretion of the enzyme in the secretion assay. This outcome was a bit counterintuitive and difficult to explain. It requires more investigation in the mechanism of gene expression from the *rmf* promoter.

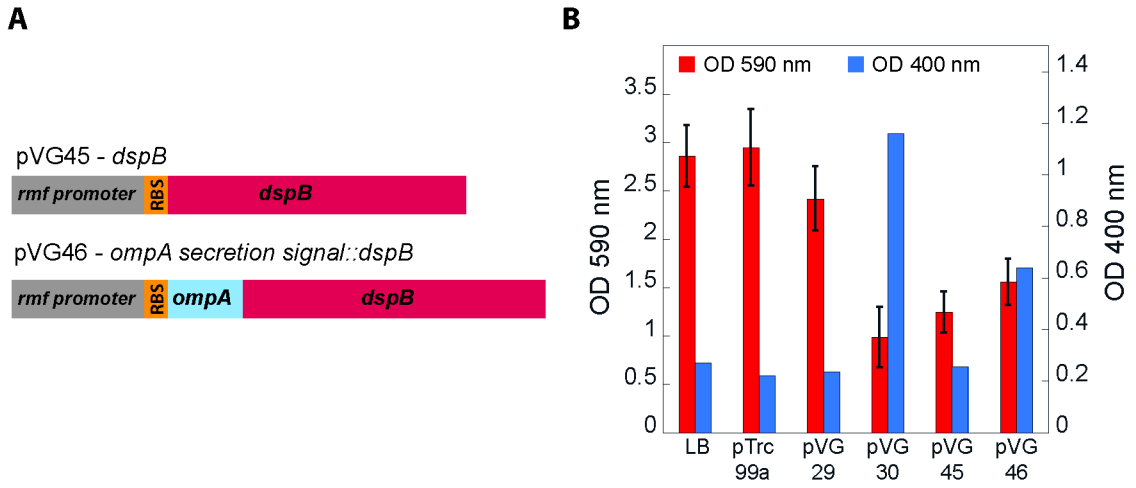

Figure 12.3. – Expressing Dispersin B from the promoter of the gene *rmf* **A**. Design of the constructs pVG45 and pVG46, **B**. Effect of the secreted enzyme on the pre-formed TRMG biofilm and the secretion assay

13. Using *agr* quorum-sensing system of *S. epidermidis*

S. epidermidis possesses a quorum-sensing system termed *agr*, in which cells respond to their own local density through a signaling molecule termed AIP (Olson *et al.* , 2014). This system could be expressed in *E. coli* that would make *E. coli* responsive to AIP made by *S. epidermidis*. Researchers have successfully exploited the quorum sensing system of *P. aeruginosa* by expressing its components in *E. coli* (Choudhary & Schmidt-Dannert, 2010).

13.1. Expressing AgrC and AgrA of *S. epidermidis* in *E. coli*

To test if the *agr* QS system can function in *E. coli*, the CDS of *agrC* and *agrA* from *S. epidermidis* RP62A was cloned in the vector ptrc99a. The resultant construct was names as pVG47.1. The intergenic region between *RNAII* and *RNAIII* of *S. epidermidis* was cloned in the vector pUA66 to generate the construct pVG48.2. As described before, cloning of any DNA fragment in the vector pUA66 enables us to measure promoter activity of that fragment. *E. coli* cells harboring both the constructs pVG47.1 and pVG48.2 were grown in the presence of 10 μM IPTG and 10 μg/ml AIP and the activity of *RNAIII* promoter was measured by flow cytometry. The activity of *RNAIII* promoter showed a small increment upon expression of AgrC and AgrA. However, there was no change observed in *RNAIII* promoter activity due to the presence of the inducers (figure 13.1). Perhaps, the proteins AgrC and AgrA were not functional in the intracellular environment of *E. coli*.

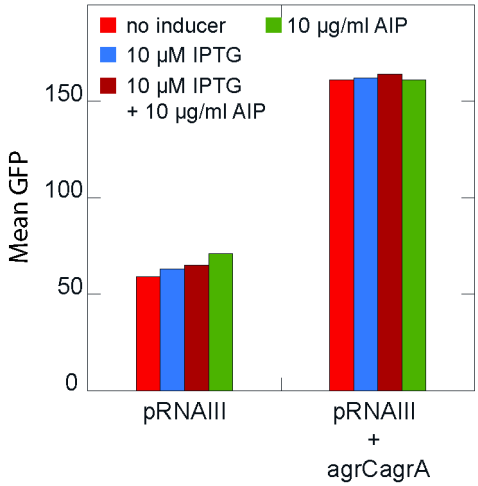

Figure 13.1. – Activity of the *RNAIII* promoter in the presence of the AIP when AgrC and AgrA of S. epidermidis are expressed simultaneously

13.2. Generating chimeric constructs of AgrA and its homologs in *E. coli*

The proteins AgrC and AgrA make a typical two-component system (TCS). AgrC is a histidine kinase that senses the presence of AIP and phosphorylates AgrA. AgrA is a response regulator, which upon phosphorylation activates the expression of *RNAII* and *RNAIII*. Members of a TCS are modular and amenable to manipulations. Domains from two different TCS proteins belonging to different species can be swapped to generate chimeric proteins, which would exhibit a combination of functions of its constituents. One such successful example is that of a chimera of Cph1 (from cyanobacteria *Synechocystis Sp.*) and EnvZ (from *E. coli*). This chimera could sense light and respond to it by transcriptional activation of a reporter through OmpR (response regulator of EnvZ) (Levskaya *et al.* , 2005). There are some other successful examples of this kind (Weerasuriya *et al.* , 1998; Michalodimitrakis *et al.* , 2005). However, this approach requires high similarity in structures, and mechanisms of signal transduction of the constituent proteins (Salis *et al.* , 2009a).

The histidine kinase AgrC belongs to a rare RHK family. The mechanism of signal transduction of AgrC is unique and there is no structural or functional homolog found in *E. coli* (Wang *et al.* , 2014). Thus, making a chimeric construct with any known histidine kinase of *E. coli* was not possible. The second possibility was to construct a chimera of AgrA and a similar response regulator of *E. coli*. AgrA is a typical response regulator with an N-terminal phosphorylation and dimerization domain, and a C-terminal DNA-binding domain. While most other response regulators share a similar architecture of DNA-binding domain, AgrA possesses a different architecture known as LytTR domain (Sidote *et al.* ,

2008). Upon surveying for other proteins with LytTR domains, two response regulators YehT and YpdB from *E. coli* were found to contain the same domain. YehT is a response regulator in YehU/YehT TCS that operates in stationary phase control network. YehT activates the expression of YjiY by binding to its promoter (Kraxenberger *et al.* , 2012). YpdB is a response regulator in YpdA/YpdB TCS that responds to the presence of pyruvate in the medium. The promoter of *yhjX* is the only target of YpdB (Fried *et al.* , 2013). It was proposed that the chimeras of phosphorylation domain of AgrA, and DNA-binding domain of YehT and YpdB each, could be phosphorylated by AgrC and activate the transcription from their cognate promoters. Eventually, the cell would be able to respond to AIP produced by *S. epidermidis*.

To test the proposed idea, chimeric DNA fragments of AgrA (aa 1-127) and YehT (aa 137-239) and YpdB (aa 139-244) respectively were made by using overlap extension PCR. The resultant fragments were cloned in the vector pTrc99a to make final constructs pVG47.2 and pVG47.3 respectively. Upstream DNA fragments of *yhjX* (cognate promoter of YpdB) and *yjiY* (cognate promoter of YehT) were cloned in the vector pUA66 to generate the constructs pVG49 and pVG50. The construct pVG47.2 was co-transformed with pVG49, and pVG47.3 with pVG50. The activation of respective promoters was tested in the same way as stated above. Unfortunately, there was no detectable expression observed from the promoter of *yhjX* (data not shown). The activity of the *yjiY* promoter showed a significant increase when the TCS was expressed in the same cell. However, there was no difference in *yjiY* promoter activity upon addition of any of the inducers (figure 13.2).

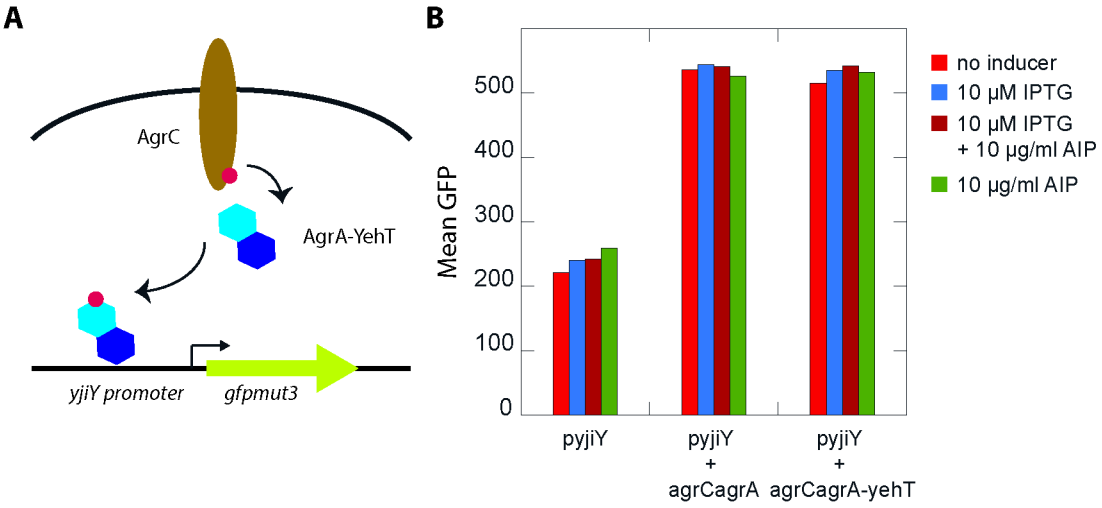

Figure 13.2. – **A**. Design of the system involving the chimera of AgrA and YehT, **B**. Activity of the *yjiY* promoter in the presence of the AIP when the chimeric protein AgrA-YehT is expressed simultaneously

Part IV.

Discussion

14. Objective and strategy

Biofilms are aggregates of bacteria embedded in the self-produced matrix. The biofilm-lifestyle is a unique feature of many bacteria, which enables them to survive through harsh environmental conditions. For many years after the discovery of bacteria, it was considered that they exist in a free-swimming or floating lifestyle. Most of the research concerning bacteria was also conducted on the basis of this assumption. As the field of microbiology grew further, it was observed that most bacteria exist in the form of biofilms.
Bacteria in the form of biofilms cause various infections in humans, animals, and plants. Bio fouling in industrial settings is caused by biofilms and it leads to huge economic loss. Researchers have attempted to develop different strategies to eradicate notorious biofilms. However, the antibacterial agents that were effective against free-swimming or floating bacteria are not equally effective in combating biofilms.
Biofilms are exceptionally resistant to the conventional methods of disinfection. This gives rise to the need of developing novel and efficient methods of combating biofilms. Among various methods tried and tested so far, biological methods have proved to be an attractive choice. Bacteria, being simple and robust in their functioning, can be engineered to perform a certain task. They can be engineered to disrupt a biofilm, which is otherwise difficult to eradicate. This strategy can particularly be helpful in treating infectious diseases where physical or chemical methods have not been successful. Thus, the objective of this thesis was to design an engineered bacterial system that can efficiently disrupt a target biofilm.
The strategy to accomplish this objective was planned in the following steps.

1. Choosing the '*killer*' and the '*victim*'
2. Choosing the '*weapon*', i.e. an anti-biofilm agent that can be expressed in the '*killer*' and can disrupt the '*victim*' biofilm
3. Choosing a suitable signaling pathway that can detect the presence of the '*victim*' biofilm and synthesize the '*weapon*' in response to it

15. Choosing the '*killer*' and the '*victim*'

The first and foremost task was to select an organism, which can be engineered as '*killer*'. *Escherichia coli* is the most studied bacterium. *E. coli* has been used as a chassis to design various biological systems involving genetic engineering. Thus, I decided to use *E. coli* for this project.
The next step was to select the '*victim*'. *E. coli* biofilms are well-studied and a great deal of information is known (Beloin *et al.* , 2008). Certain strains of *E. coli* are pathogenic, and they are similar to the WT strains used in the lab. A system that can work against *E. coli* biofilms can be modified and used against other similar pathogenic bacteria. Thus, I decided to consider *E. coli* biofilm as one of the targets to design the proposed system.
Staphylococcus epidermidis is known to make biofilms on indwelling devices and cause infections that are difficult to treat. The process of biofilm formation by *S. epidermidis* and its composition has been studied well in detail (Otto, 2008). Developing a strategy against a pathogenic organism has better prospects. Thus, I chose *S. epidermidis* biofilm as another '*victim*' to demonstrate the action of the proposed system.

15.1. Selecting Dispersin B as an anti-biofilm agent

The second step was to choose the most effective method of biofilm disruption that is compatible with the engineered bacterial system. Most of the commonly used disinfectants were artificially synthesized compounds that could not be synthesized biologically. Some inhibitors of metabolism or signaling that were successfully used against bacterial biofilms could be synthesized biologically. However, their synthesis involved many steps and it was difficult to engineer bacteria for such a complicated synthesis process. Considering the limitations of other biochemical agents, matrix-degrading enzymes were an attractive choice. The only enzyme that was shown to be effective against *E. coli* biofilms as well as *S. epidermidis* biofilms was Dispersin B. Dispersin B cleaves β, 1⇒6 glycosidic bond that links NAG residues in poly-N-acetyl glucosamine (PGA) found in the matrix of *E. coli* biofilms (termed as PIA or PNAG in *S. epidermidis*). This enzyme was a product of a single gene that could be easily overexpressed in *E. coli*. Its synthesis involved a single step, and it was not toxic to *E. coli* when overexpressed. Therefore, I decided to use this

enzyme as a '*weapon*' in the proposed system.

15.2. The action of Dispersin B on WT *E. coli* biofilms

I tested the activity of purified Dispersin B on the pre-formed biofilms of various WT strains of *E. coli*. It was observed that the effect of the enzyme was not significant on WT *E. coli* biofilms. However, when the biofilms of these strains were grown in the presence of Dispersin B, there was inhibition of biofilm formation (figure 9.2). In *E. coli* biofilms, the polymer PGA is required for the transition of cells from temporary to permanent attachment (Agladze *et al.* , 2005). The polymer is strongly associated with cells and mostly localized at the cell poles (Itoh *et al.* , 2008). Along with PGA there are other components in the biofilm matrix, such as Curli fibers, Type 1 fimbriae and cellulose. The relative abundance of these components in the matrix depends on the kind of the strain and the conditions of biofilm formation. All the components of the matrix are collectively responsible for the structural integrity of the biofilm. Their relative contribution to the integrity of the biofilm could be different in different strains and also varies according to the conditions of growth. In the given experimental setting, it was observed that only *E. coli* MG1655 contained PGA as an important component contributing to the integrity of the biofilm. The biofilms formed by this strain were, however, less robust than other WT strains. In case of other WT strains of *E. coli*, Dispersin B could not disrupt their pre-formed biofilms. However, it inhibited their biofilm formation to varying extent. This implies that these strains also contain PGA in their matrix and it is indeed involved in the initial phase of biofilm formation. However, once the biofilm is formed, degrading PGA does not significantly affect its structural integrity.

15.3. Selecting the biofilm of *csrA* mutant of *E. coli* as a '*victim*'

CsrA (carbon storage regulator) is a global regulator in *E. coli* that regulates many unrelated functions in a post-transcriptional manner. CsrA plays an important role when cells switch from the motile planktonic phase to sessile biofilm lifestyle. It drives the cell more towards the motile planktonic phase and reduces its likelihood of acquiring the biofilm-lifestyle. It binds to the 5' untranslated region of *pgaABCD* mRNA, and inhibits its translation. This results in the reduction of PGA synthesis. The *csrA* mutant used in this thesis encodes the protein CsrA that is defective in its action of binding to the target mRNA. The strain makes excess of PGA, and makes prolific biofilms. This mutation mim-

ics that state of the cell, in which the cell is switching to the biofilm-lifestyle. Dispersin B could efficiently disrupt the robust biofilms made by the *csrA* mutant (figure 9.3). This observation proved that the biofilm of *csrA* mutant (*E. coli* TRMG1655) was an attractive choice as a '*victim*'.

15.4. Secretion of Dispersin B

Various strategies have been explored to drive a recombinant protein out of the *E. coli* cell. I decided to use the simplest method of fusing a secretion signal sequence of OmpA to the N-terminus of Dispersin B. The secretion signal sequences from some other proteins like FlgM and YebF were also tested. However, use of these signals was not successful in driving Dispersin B out of the cell.

The presence of the secreted enzyme was detected by growing the cells in a medium containing the substrate NP-GlcNAc. The presence of OmpA secretion signal sequence could successfully drive the enzyme out of the cell. The expression and secretion of Dispersin B was extremely slow. To observe at least 50 % reduction in the '*victim*' biofilm, the '*killer*' cells had to be incubated on top of the pre-formed '*victim*' biofilm for at least 12 hours. This time span was too large in comparison to hardly one hour of incubation required by the purified enzyme. The 'killer' cell was incubated on the pre-formed biofilm at 26 °C, without shaking. Under these conditions, *E. coli* cells grow slow and hence the rate of the protein production is also slow. The protein OmpA is transported across the inner membrane in Sec-dependent manner. The rate of transport of OmpA-Dispersin B depends on the number of Sec proteins available in the cell. Consequently, not all the enzyme that is synthesized inside the cell is transported out of the inner membrane. After crossing the inner membrane, the enzyme has to cross another barrier of the outer membrane. Limited numbers of the outer membrane secreton proteins further reduce the rate of transport of the enzyme out of the cell. The enzyme activity was also detected in the periplasmic fraction of the 'killer' cells (data not shown) indicating that there was an accumulation of Dispersin B in the periplasm. This explains why the 'killer' cell required long time of incubation to disrupt the 'victim' biofilm.

16. Using the N-Acetyl glucosamine signaling pathway in *E. coli*

The next step in accomplishing the objective of this project was to select the right sensory pathway. I proposed three different strategies to engineer the '*killer*' so that it detects the '*victim*' biofilm and synthesizes Dispersin B as a response to the detection. The first strategy involves using the N-acetyl glucosamine signaling pathway in *E. coli*.
N-acetyl glucosamine (NAG) is the constituent of PGA, which is the prominent component of the matrix of the '*victim*' biofilm. It was the most attractive choice for a signaling molecule. *E. coli* possesses a set of genes that encode proteins involved in the uptake and metabolism of NAG. These genes are expressed when the cell detects the presence of NAG in the environment. Combining NAG metabolism with Dispersin B synthesis would generate a 'biofilm sense and destroy' system. The '*killer*' would keep producing Dispersin B as long as there is sufficient supply of NAG. Thus, I decided to use *nagE* promoter of *E. coli* to drive the synthesis of Dispersin B.

16.1. NAG generated from the action of Dispersin B was too low to activate the *nagE* promoter

In order to design the proposed system, it was essential to characterize how the *nagE* promoter responds to extracellular NAG. The analysis of *nagE* promoter activity showed that the *nagE* promoter required 50 µM of extracellular NAG to induce two-fold increase in its activity. The induction of the *nagE* promoter was rather low in the presence of 1 to 10 µM of extracellular NAG. The concentration of NAG in the '*victim*' biofilm supernatant was estimated by MBTH assay. It was less than 1 µM. The procedure of biofilm supernatant preparation might not have isolated PGA in its entirety. Therefore, the results of the MBTH assay were thought to be an underestimate. The same biofilm supernatant was used to test the activity of the *nagE* promoter. Unfortunately, it could not activate the *nagE* promoter above its basal expression level.
To circumvent the issue of dilution of NAG in the supernatant, the reporter cells harboring

the construct pVG18 were incubated with the Dispersin B-treated '*victim*' biofilm and the activity of the *nagE* promoter was estimated by flow cytometry. Here, the reporter cells were in the presence of the unprocessed biofilm and hence NAG generated from the biofilm was in direct contact with the cells. In this experiment the reporter cells had low basal expression of GFP. It was difficult to distinguish them from the cells of the '*victim*' biofilm while interpreting flow cytometry data. However, there was no obvious increase observed in the activity of the *nagE* promoter.

16.2. Overexpressing the *pgaABCD* operon was insufficient to elevate the levels of PGA

In *E. coli*, PGA is strongly associated with the cell surface and localized mainly to the cell poles (Wang *et al.* , 2004). Once the cells are permanently attached, the expression of PGA is perhaps not continued. As a result, the amount of PGA in the biofilm matrix with respect to other components is not too high. In fact, researchers have found that in the biofilm matrix of *E. coli* O157:H7, NAG is found to be hardly 1.9% of the total carbohydrates (Bales *et al.* , 2013). Though there can be variation in the biofilm matrix of different strains, this number cannot be expected to have significant variation. The mutation in *csrA* increased the synthesis of PGA. This increase was enough to make a substantial change in the morphology of the biofilm. The mutation also made PGA as the most prominent component of the biofilm matrix. However, it could not result in generating the amount of NAG that was necessary to activate the *nagE* promoter.
To elevate the levels of PGA synthesized by the '*victim*' biofilm, entire *pgaABCD* operon was overexpressed in the *csrA* mutant of *E. coli*. However, the highest level of overexpression of *pgaABCD* operon resulted in less than 2.5 fold increase in biofilm formation. The biofilm overexpressing pgaABCD operon was treated with Dispersin B and the activation of the *nagE* promoter was assessed by incubating the reporter cells on top of the treated biofilm. Unfortunately there was no significant increase observed in *nagE* promoter activity. Although overexpressing *pgaABCD* operon resulted in increasing PGA production, the hydrolysis of PGA into NAG was not sufficient to induce the *nagE* promoter. The level of PGA production depends not just on the level of expression of enzymes involved in its synthesis, but also on other factors. For example, cyclic-di-GMP generated from the action of DgcZ is necessary for the assembly of the export machinery of PGA (Steiner *et al.* , 2013). Perhaps the action of DgcZ was limiting the overproduction of PGA. Therefore, despite overexpressing the entire pgaABCD operon there was less than 2.5 fold increase in biofilm formation. Due to limited amount of PGA, its hydrolysis could not generate high concentration of NAG, and the expected increase in the activity of the *nagE* promoter was not observed.

Although the amount of PGA synthesized by cells was low, the action of Dispersin B was able to disrupt the '*victim*' biofilm completely. This implies that PGA, although present in little amount was important for the structural integrity of the '*victim*' biofilm. Ideally, the action of purified Dispersin B should have generated many molecules of NAG after complete hydrolysis of the polymer. However, the difference in the concentration of NAG between Dispersin B treated and untreated samples, as observed by MBTH assay, was too low. It indicates that the action of Dispersin B did not hydrolyze the entire polymer. The reason behind this could be the exolytic action of the enzyme (Kerrigan *et al.* , 2015). Hydrolysis of a few residues of the polymer was sufficient to disrupt the structural integrity of the biofilm. However, it did not generate enough molecules of NAG. Researchers came across a similar observation in case of *S. epidermidis* biofilms (Goekcen *et al.* , 2013). They observed that Dispersin B at a concentration as low as 1 μg/ml could efficiently disrupt *S. epidermidis* biofilms. However, to see a detectable amount of NAG the concentration of Dispersin B needed was as high as 1000 μg/ml.

16.3. Sensitivity of the *nagE* promoter could not be improved

Attempts were made to improve the sensitivity of the *nagE* promoter by tweaking the levels of the components of the *nagE/BACD* operon. The idea behind this was to alter the basal levels of NAG-6-P inside the cell so that the cell becomes more sensitive to extracellular NAG. Overexpression of NagC (the repressor) reduced the activity of the *nagE* promoter at all the concentrations of extracellular NAG. Overexpressing the catabolic enzymes NagB and NagA resulted in the same outcome. When NagE (the transporter of NAG) was overexpressed, it resulted in the increase in the activity of the *nagE* promoter for all concentrations of extracellular NAG. In any of these attempts, the fold-change in the activity of the *nagE* promoter in the range of 1 to 10 μM of extracellular NAG was unaffected.

The enzymes encoded by the genes in the *nagE/BACD* operon play an important role in peptidoglycan recycling. When cells are dividing, they are constantly breaking down and synthesizing peptidoglycan, which is a component of the cell wall. Since NAG is a major constituent of peptidoglycan, there is always some level of NAG-6-P present inside the cell. Although enzymes encoded by the genes in the *nagE/BACD* operon have a major contribution in NAG metabolism, there are alternative pathways present in the cell, which compensate for the loss or overexpression of genes in the *nagE/BACD* operon. For example, NagK phosphorylates NAG generated from the hydrolysis of peptidoglycan oligomers and makes NAG-6-P. This action maintains the basal level of NAG-6-phosphate inside the cell when NagB and NagA are overexpressed. In short, metabolism of NAG

quickly adjusts itself in such a way that any perturbations in its components would result in minimal change in the levels of NAG metabolites inside the cell. These perturbations could have a detectable effect on the activity of the *nagE* promoter at higher concentrations of extracellular NAG. However, the proposed system required that the *nagE* promoter is able to detect extremely low concentration of NAG generated from the '*victim*' biofilm. Thus, tweaking the levels of the enzymes involved in NAG metabolism was ineffective for the proposed system.

Apart from the low amount of PGA in the '*victim*' biofilm and low sensitivity of the *nagE* promoter, there was another hurdle in making the proposed system work. It was the mismatch between the time required for the expression and secretion of Dispersin B and the time required for the activation of the *nagE* promoter. The activity of the *nagE* promoter at 100 μM of extracellular NAG, started declining within ~ 90 minutes after the addition of NAG (figure 10.1). This was the case when the cells were growing exponentially in the presence of NAG at 37 °C and shaking at 200 rpm. The decline in the activity was mainly due to the presence of the catabolic enzymes NagB and NagA. As described in section 4.1, the expression of the catabolic enzymes NagB and NagA is also up regulated when the cell takes up NAG from the environment. The increasing catabolic activity of these enzymes results in the complete utilization of NAG and decline in the activity of the *nagE* promoter. Since the enzymes of NAG metabolism are involved in peptidoglycan recycling, their turnover is higher is fast-growing cells. Hence, the transient activation of the *nagE* promoter was observed. In the conditions of biofilm disruption (26 °C and without shaking) the 'killer' cells grow slow. Hence, the change in the activity of the *nagE* promoter was found to be much lesser and slower than that observed in the former condition (data not shown). As evident from figure 9.5, the '*killer*' cells required at least 12 hours of incubation to secrete Dispersin B that brought about 50% disruption of the '*victim*' biofilm. Thus, even if the *nagE* promoter had been sensitive enough to detect the low amount of NAG generated from the '*victim*' biofilm, driving the expression of Dispersin B from the induction of NAG would have been difficult to execute.

17. Using chemotaxis to enhance the efficiency of the system

Combining chemotaxis with the proposed system was indeed an attractive strategy. By using chemotaxis, the '*killer*' specifically migrates towards the '*victim*' biofilm and secretes Dispersin B only in the vicinity of the '*victim*' biofilm. The fact that *E. coli* is chemotactic towards NAG was known (Adler *et al.* , 1973). However, its exact mechanism was not explored. In this project, it was found that the chemotaxis towards NAG is PTS mediated and not receptor mediated. Also, it was observed that the range of concentration within which *E. coli* shows chemotaxis towards NAG is between 1 to 10 μM. Since the concentration of NAG at some distance away from the biofilm would be much lower than this range, it was necessary that the '*killer*' shows chemotaxis towards NAG at a relatively lower concentration.

For the PTS-mediated chemotaxis pathway, the protein involved in binding to the extracellular sugar is the transporter of that sugar. It is the uptake of the sugar through the transporter initiates the cascade of reactions that eventually lead to chemotaxis. In case of chemotaxis towards NAG, it is the transporter NagE, which performs this important role. In order to improve the chemotaxis towards NAG, the transporter NagE overexpressed and the chemotactic response of the cells was studied by stimulus-dependent flow-FRET. The modern biophysical technique of flow-FRET allowed the quantitative analysis of the chemotactic response. NagE being a membrane protein, its overexpression in the WT cells resulted in slower growth and lack of fluorescent signal in the flow-FRET setup. Hence, the transporter was overexpressed in the deletion mutant of the transporter itself. Unfortunately, there was no change in the range of the concentration within which the cells show chemotaxis towards NAG.

The PTS-mediated pathway of chemotaxis is not as modular as the receptor-mediated pathway. In fact, the exact mechanism of interaction of PTS proteins with the chemotactic proteins is not completely understood. The only aspect that is understood so far is that PtsI directly interacts with CheA and influences its kinase activity (Neumann *et al.* , 2012). The possible reason behind the above observation is that that the amount of PtsI present in the cell is limited. More transporters bring more NAG inside the cell, however there are no sufficient levels of PtsI present in the cell to transduce the signal. This speculation required further experiments, which was beyond the scope of this thesis.

18. Using biofilm-specific gene expression

The second strategy is about exploiting the biofilm-specific gene expression in *E. coli* to drive the synthesis of the '*weapon*'. The biofilm mode of lifestyle is fundamentally different from the planktonic lifestyle. The phenotypic characteristics of biofilms are thought to be regulated by a specific pattern of gene expression. In the perspective of this project, such biofilm-specific up regulation of genes can be regarded as a signaling pathway. Although the presence of any signaling molecule is debatable, the output of this imaginary signaling pathway is certainly attractive for this project. If such a gene(s) is found, the promoter associated with it can be used to drive the expression of Dispersin B. The '*killer*' will approach the '*victim*' biofilm, get incorporated into the biofilm and start expressing the enzyme once it acquires the lifestyle of a biofilm. This will eventually disrupt the biofilm from within.

Researchers have explored into the biofilm-specific gene expression by DNA microarray-based techniques. However, there is no unanimous outcome of different experiments. The observations from the previous studies imply that the pattern of gene expression depends on the conditions in which biofilms were grown. Thus, I thought of exploring the pattern of gene expression in *E. coli* biofilms grown under conditions relevant to the objective of this project. I used the most advanced and sensitive method of RNA-seq. The transcriptome of the planktonic cells and the attached cells of the WT *E. coli* biofilms was compared with that of the exponential phase cells of the same. It was found that most of the genes up regulated in attached cells were also up regulated in planktonic cells. However, these findings were in agreement with the previous work (Beloin *et al.* , 2004). Most of the genes that were found to be up regulated in biofilms encode proteins involved in metabolic processes such as glycolate metabolism, fatty acid oxidation, oxidoreductase activity, and others. There were also some genes, which are involved in responding to extracytoplasmic stress (*cpxP*), regulation of capsule synthesis (*rcsB*), regulation of curli assembly (*csgD*) and some other regulatory functions.

The data were further sorted to find those genes, which are up regulated only in attached cells and not in planktonic cells of the biofilm with respect to the exponential phase cells. A set of such genes was shortlisted and their corresponding promoters were cloned upstream to the CDS of gfp to make the reporter constructs. These reporter constructs were then

tested for protein synthesis under the same conditions, in which cells were grown for RNA isolation. Most of these reporter constructs did not show any detectable expression. This could be due to the lack of regulatory elements in the DNA fragments used to make these constructs.

Planktonic cells of the biofilm are those cells, which are either dispersed from the biofilm or not attached at all. In the given experimental setting, they are floating in the close vicinity of the biofilm. It was possible to drive the expression of Dispersin B from one of the planktonic-phase promoters. The enzyme could diffuse to the biofilm and disrupt the biofilm. In order to find such a promoter, the genes up regulated in planktonic cells were further sorted and a few of the genes were selected. The activity of the promoters associated with these genes was measured in different phases of the cells.

The promoter of the gene *rmf* showed high expression in planktonic cells of the biofilm. This gene encodes a 'ribosome modulating factor' that is overexpressed when the cells enter stationary phase. Rmf binds to 70S ribosomal subunit inducing its dimerization that in turn inhibits the binding of the ribosome to mRNA. Thus, overexpression of Rmf results in slowing down of translational activity of the cell. As cells acquire the biofilm-lifestyle they show slowed growth rate and metabolic activity (Wada *et al.* , 1995). The metabolic state of a cell in a biofilm is quite similar to that in a stationary phase. Thus, it was intuitive to find the overexpression of Rmf in the planktonic cells of a biofilm. Although the overexpression of Rmf was not biofilm-specific, I decided to use the promoter of the gene *rmf* to drive the expression of Dispersin B. The '*killer*' cells could successfully synthesize and secrete Dispersin B when expressed from the *rmf* promoter. However, the cells carrying the constructs with and without the secretion signal did not show any significant difference in their activity on the biofilm. This outcome was surprising and difficult to explain. As shown in figure 12.3, the constructs with and without the secretion signal show the expected difference in the secretion of the enzyme. The secretion assay was performed by growing the cells in the M9 minimal medium. To observe the change in the color of the substrate, a transparent medium is necessary. The LB medium, being yellow in color is unsuitable for the secretion assay. When the cells were used to disrupt the 'victim' biofilm, they were grown in the LB medium to achieve higher growth rate and higher enzyme production. It is possible that the growth of the cells in the LB medium had an effect on the way genes are expressed from the *rmf* promoter. In the late stationary phase, when the *rmf* promoter is activated, the cells are perhaps leaky (Casadei *et al.* , 2002). They tend to release some proteins regardless of the presence of the secretion signal. This speculation, however, requires further investigation.

19. Using the *agr* quorum-sensing system of *S. epidermidis*

S. epidermidis harbors a quorum-sensing system called *agr* (accessory gene regulator) system by which, the cells respond to their own local density via a signaling molecule called AIP (auto-inducing peptide). Cells within the biofilm of *S. epidermidis* regulate the expression of many genes through this system. Thus, the signaling molecule AIP was an attractive molecule to be exploited to detect the presence of the biofilm. Components of the quorum-sensing system in Pseudomonas aeruginosa have been successfully expressed in *E. coli* (Choudhary & Schmidt-Dannert, 2010). On the similar lines it was proposed that the *agr* system of *S. epidermidis* could be expressed in *E. coli* to detect the presence of the *S. epidermidis* biofilm. To test this hypothesis, the promoter that responds to the quorum sensing system was cloned upstream to the CDS of GFP to make a reporter construct and the components involved in sensing were co-expressed along with it. Unfortunately, the reporter construct did not respond to the AIP molecules in the medium. The components of the *agr* system were not functional in *E. coli*.

Generating chimeric proteins is an excellent strategy to combine the properties of two different proteins. The protein AgrA of *S. epidermidis* shared sequence and domain-organization similarity with two proteins of *E. coli*, YehT and YpdB. Thus, it was proposed to generate chimeric proteins containing phosphorylation domain of AgrA and DNA-binding domain of YehT and YpdB respectively. The promoters associated with YehT and YpdB were cloned to make reporter constructs. The reporter construct of *yjiY* (cognate promoter of *yehT*) showed an increase in its activity when the chimeric protein AgrA-YehT and AgrC were expressed. However, there was no response observed to the AIP. Generating functional chimeric proteins has been successful in some lucky instances where the two parent proteins had a striking similarity in the structure and the mechanism of signal transduction. In this case, both YehT and YpdB contain a LytTR domain that is also found in AgrA (Sidote *et al.* , 2008). The function of this domain is to bind a specific sequence of DNA and activate the gene expression. However, the ability of this domain to bind DNA depends on the phosphorylation state of the other domain (in this case, CheY-like domain). Perhaps, it is the phosphorylation-induced change in the conformation of the protein that dictates the DNA-binding ability of the LytTR domain. This conformational change can be different for different proteins. Generating chimeras of two

proteins with different patterns of the conformational change may not result in functional chimeric protein. In this case, the exact pattern of the conformational change is unknown. Thus, it is difficult to generate a functional chimera of these proteins.
Apart from the functionality of the chimeric proteins, the functionality of the receptor AgrC also needs to be considered. AgrC is a membrane bound histidine kinase, which senses the presence of the AIP and phosphorylates AgrA. According to the structure and the mechanism of signal transduction, AgrC belongs to a rare family of proteins. It was possible that its functioning required the microenvironment of the cell membrane of a typical Gram-positive bacterium. Since the receptor itself was perhaps not functional in *E. coli*, it was unlikely that *E. coli* could respond to the AIP.

20. Conclusion and future prospects

The objective of this thesis was to design a biologically engineered system to combat bacterial biofilms. I have successfully shown that a matrix-degrading enzyme- Dispersin B can be an efficient *'weapon'* to destroy the target biofilm. The enzyme can be easily overexpressed and secreted by the '*killer*' cell. The next step in accomplishing the goal of this project was to combine the expression of Dispersin B with a suitable signal transduction pathway. I attempted to exploit the NAG metabolic pathway, the NAG chemotaxis pathway and biofilm-specific gene expression to achieve this goal. Alternatively, I designed a similar system to disrupt the biofilm of *S. epidermidis*. In this system, I attempted to use the *agr* quorum sensing system to engineer the '*killer*' that can disrupt *S. epidermidis* biofilms.

In the course of accomplishing the objective of this project, I have explored the metabolic pathway of NAG and the chemotactic pathway of NAG in *E. coli*. Although certain facts were already known about these pathways, I have unraveled some interesting features of these pathways. The chemotactic pathway of *E. coli* is sensitive to extracellular NAG within the range of 1 to 10 μM. Whereas for the catabolic pathway of NAG, the same range of concentration is insignificant. The *nagE* promoter shows a significant increase in its activity only beyond 10 μM of extracellular NAG.

While demonstrating the effect of Dispersin B on *E. coli* biofilms, I have indirectly investigated the relative importance of PGA in biofilm formation. It was observed that PGA becomes critical to the structural integrity of the biofilm only when it is synthesized in excess. In the WT strain of *E. coli*, PGA is important only in initial stages of biofilm formation. Once the mature biofilm is formed other components of the matrix become more important than PGA.

Exploring the biofilm-specific pattern of gene expression has been the most interesting task performed in this project. The general trend observed in the genes overexpressed in biofilms was more or less in agreement with previous studies. Considering great differences in opinions shared by scientists regarding gene expression in biofilms, this observation is remarkable. The genes that were found to be up regulated only in attached cells but not in planktonic cells encode for enzymes involved in combating oxidative stress. It is counter-intuitive to see oxidative stress in the deeper layers of a biofilm. Perhaps it is an indirect effect that takes place through an unknown protein. It is certainly worth exploring

this question further.

In the context of the bacterial species used in this thesis, the proposed system was only partially successful. However, the idea underlying this project is extremely versatile and still holds a possibility of being successful in some other species of bacteria. The idea requires finding an abundant and important component of a biofilm matrix, an enzyme that degrades this component and a signaling pathway that responds to the product of the action of the enzyme. For example, the biofilm matrix of *P. aeruginosa* contains alginate that is essential for its structural integrity. The enzyme alginate lyase is known to degrade alginate and generate uronic acids as products of its action. It is possible to engineer *E. coli* to express alginate lyase from the promoter associated with uronic acid metabolism. The '*killer*' cell would continue producing the enzyme as long as there are enough uronic acids being generated from the biofilm. The same module can be extended to any other bacteria for which the above details are known. The concept of exploiting biofilm-specific gene expression does not even require the presence of a specific metabolic pathway. Identification of a gene that is multi-fold up regulated upon acquiring a biofilm-lifestyle is sufficient for extending the model to other species of bacteria.

Appendix

List of strains

Following is the list of strains used in this study (Table 20.1).

Table 20.1. – List of strains

Name of the strain	Genotype	Antibiotic Resistance	Source and reference
E. coli MG1655	F^- λ^- ilvG$^-$ rib-50 rph-1	none	Received from G. Krammer (ZMBH, University of Heidelberg) (Hayashi *et al.* , 2006)
E. coli K12 W3110	F^- λ^- INV(rrnD, rrnE) rph-1	none	Received from G. Kramer (ZMBH, University of Heidelberg) (Hayashi *et al.* , 2006)
E. coli K12 W3110 *ΔfliC*	F^- λ^- INV(rrnD, rrnE) rph-1 *ΔfliC*	none	Received from Verena Suchanek
E. coli K12 LJ110	W3110, F^-, Fnr^+	none	(Zeppenfeld *et al.* , 2000)
E. coli K12 LJ110 *ΔnagE*	W3110, F^-, Fnr^+, *ΔnagE*	Kan	This work
E. coli K12 LJ110 *ΔptsIΔcpdA*	W3110, F−, Fnr+, *ΔptsIΔcpdA*	Kan	Dr. Karin Grosse
E. coli UU1250	RP437 *Δaer*-1 *ygjG::Gm* *Δtsr*-7028 *Δ(tar-tap)*5201 *zbd::Tn5* *Δtrg*-100 thr+met+	none	(Ames *et al.* , 2002)

E. coli K12 W3110 RH	F^- λ^- INV(rrnD, rrnE) rph-1	none	Received from Dr. Regine Henge (Humboldt University, Berlin) (Hayashi *et al.* , 2006)
E. coli K12 W3110 AR	F^- λ^-IN(rrnD-rrnE) rph-1	none	Received from Dr. Regine Henge (Humboldt University, Berlin) (Serra *et al.* , 2013)
E. coli M15 (contains pREP4)	F^-, Φ80ΔlacM15, thi, lac^-, mtl^-, $recA^+$, KmR	Kan	Quiagen
E. coli TRMG1655	*E. coli* K12 MG1655 *csrA::kan*	Kan	Received from Dr. Tony Romeo (University of Florida) (Romeo *et al.* , 1993)
E. coli DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZΔM15* *Δ(lacZYA-argF)*U169, hsdR17(r_K^- m_K^+), λ^-	none	Invitrogen
S. epidermidis RP62A		none	ATCC

List of plasmids

Following is the list of plasmid constructs made in this study (Table 20.2)

Table 20.2. – List of plasmids

Name of the Plasmid	Fragment cloned	Cloning vector	Antibiotic resistance
pVG17	*nagE*	pBAD33	cam
pVG18	*nagE promoter::GFP*	pUA66	kan
pVG21	*ompA::dspB*	pQE60	amp
pVG22	*dspB*	pQE60	amp
pVG23	*nagC*	ptrc99a	amp
pVG24	*nagB nagA*	pBAD33	cam
pVG29	*dspB*	ptrc99a	amp
pVG30	*ompA::dspB*	ptrc99a	amp
pVG31	*rmf promoter*	pUA66	kan
pVG32	*prpB promoter*	pUA66	kan
pVG33	*wza promoter*	pUA66	kan
pVG35	*csrB promoter*	pUA66	kan
pVG36	*aceB promoter*	pUA66	kan
pVG37	*astC promoter*	pUA66	kan
pVG38	*cysJ promoter*	pUA66	kan
pVG39	*cpxP promoter*	pUA66	kan
pVG40	*rpoS promoter*	pUA66	kan
pVG41	*pgaABCD*	pBAD33	cam
pVG42	*pgaABCD*	pBAD24	amp
pVG45	*prmf::RBS2::dspB*	ptrc99a	amp
pVG46	*prmf::RBS2::ompA::dspB*	ptrc99a	amp
pVG47.1	*agrC agrA* of *S. epidermidis*	ptrc99a	amp
pVG47.2	*agrC agrA::ypdB*	ptrc99a	amp
pVG47.3	*agrC agrA::yehT*	ptrc99a	amp
pVG48.2	*RNAIII promoter*	pUA66	kan
pVG49	*yhjX promoter*	pUA66	kan
pVG50	*yjiY promoter*	pUA66	kan
pVG51	*fim promoter*	pUA66	kan
pVG52	*fad promoter*	pUA66	kan
pVG53	*trxC promoter*	pUA66	kan
pVG54	*grxA promoter*	pUA66	kan
pVG55	*oxyS promoter*	pUA66	kan

pVG56	*ahpC promoter*	pUA66	kan

Following is the list of cloning vectors and other plasmids used in this study (Table 20.3)

Table 20.3. – List of cloning vectors

Name of the Plasmid/Vector	Specification	Antibiotic resistance	Source and reference
pBAD24	ori pBR322, P_{BAD} promoter	Amp	Guzman *et al.* , 1995
pBAD33	ori pBR322, P_{BAD} promoter	Cam	Guzman *et al.* , 1995
pQE60	ori ColE1, P_{TAC} promoter	Amp	Quiagen
pTrc99a	ori pBR322, P_{TAC} promoter	Amp	Amann *et al.* , 1988
pUA66	ori Sc101, *gfpmut3*	Kan	Zaslaver *et al.* , 2006
pJK618	*dspB*	Amp	Kaplan *et al.* , 2003
pVS88	*cheY-yfp cheZ-cfp*	Amp	Sourjik *et al.* , 2007

List of primers

Following is the list of primers used in this study (Table 20.4)

Table 20.4. – List of primers

Name of the Primer	Sequence	For the amplification of	Restriction site
VG17f	ATCGAGCTCTCGTAGGGGGAATA AGATGA	*nagE* CDS of *E. coli* with RBS	SacI
VG17r	GTCTCTAGATTACTTTTTGATTTCAT ACAGCGG	*nagE* CDS of *E. coli* with RBS	XbaI
VG18f	TGACTCGAGTGCGCAGACAGGCG TCAATC	*nagE* promoter of *E. coli*	Xho1
VG18r	CGTGGATCCCTTATTCCCCCTACG AGAACCCTATTTG	*nagE* promoter of *E. coli*	BamH1

VG23f	AAATTACCATGGGAATGAAAAAAAC CGCGATTGCGATTGCGGTGGCGCT GGCGGGCTTTGCGACCGTGGCGC AGGCGAAT	*dspB* with *ompA* secretion tag	Nco1
VG23rB	GTGATGAGATCTGGATCCCTCAT CCCCATTCGTCTTAT	*dspB*	BamH1
VG24f	AAATTACCATGGGAATTGTTGCG TAAAAGGCA	*dspB* without *ompA* secretion tag	Nco1
VG24r	GTGATGAGATCTGGATCCGCCTCC GCCTCCGCCCTCATCCCCATTCGT CTTAT	*dspB* with glycine linker	BamH1
VG26f	AATTCGAGCTCTGAGGTGAATAAT GAG ACTGA	*nagBA* genes of *E. coli*	Sac1
VG26r	TCGACTCTAGAGCCTGGTGTCATAC TTT CTC	*nagBA* genes of *E. coli*	Xba1
VG37f	ACTGCTCGAGTTGTAAATATAACCG TCT CCG	*rmf promoter* of *E. coli*	Xho1
VG37r	ACTGGGATCCGCCTCGTTTCCCTC ATAC	*rmf promoter* of *E. coli*	BamH1
VG38f	ACTGCTCGAGTTGTTGCAATGAAA CGCGG	*prpB promoter* of *E. coli*	Xho1
VG38r	ACTGGGATCCGCCCATCCTTTGTT ATCAACT	*prpB promoter* of *E. coli*	BamH1
VG39f	ACTGCTCGAGCAAAACCTATTCGT TGTATGAC	*wza promoter* of *E. coli*	Xho1
VG39r	ACTGGGATCCTTGTTTATTTATCAC TTTGGCAG	*wza promoter* of *E. coli*	BamH1
VG41f	ACTGCTCGAGCCATCTGGTTGTGA GAGAT	*csrB promoter* of *E. coli*	Xho1
VG41r	ACTGGGATCCCCCTGTCGACGAA GATAGAA	*csrB promoter* of *E. coli*	BamH1
VG42f	AGTCCTCGAGGTTATACCGCCAG TAATGCT	*fimA promoter* of *E. coli*	Xho1

VG42r	ACTGGGATCCTGTAGAACTGAGG GACAGAG	*fimA promoter* of *E. coli*	BamH1
VG43f	ATCGCTCGAGAACGCTGGATTAA TCTTCTGTG	*aceB promoter* of *E. coli*	Xho1
VG43r	ATCGGGATCCTTCAGTCATCGTG CAGCTC	*aceB promoter* of *E. coli*	BamH1
VG44f	ACTGCTCGAGTGTAAAGCATGTC AGTCTCC	*fadB promoter* of *E. coli*	Xho1
VG44r	ACTGGGATCCAGGCCAGTGATTC CATTTTT	*fadB promoter* of *E. coli*	BamH1
VG45f	TGACCTCGAGCAGGCCGTTGATA TTAAAAGA	*astC promoter* of *E. coli*	Xho1
VG45r	TACGGGATCCAAGTTTTCACGCG TAATTGG	*astC promoter* of *E. coli*	BamH1
VG46f	AGTCCTCGAGGAAATCTTTAAATA ACGTGGTGG	*cysJ promoter* of *E. coli*	Xho1
VG46r	TGACGGATCCGTCATGCGTCGTT ATGTTC	*cysJ promoter* of *E. coli*	BamH1
VG48f	TACGCTCGAGTCGGTCATCATCA ACTAACA	*cpxP promoter* of *E. coli*	Xho1
VG48r	ACTGGGATCCCTATGCGCATCAT TTGCTC	*cpxP promoter* of *E. coli*	BamH1
VG50f	TCGACTCGAGGATAAATCGGCG GAACCAG	*rpoS promoter* of *E. coli*	Xho1
VG50r	ACTGGGATCCCTGCGATAACAGT TCCTCTT	*rpoS promoter* of *E. coli*	BamH1
VG51fk	GCTCGGTACCCAATACATGGAGT AATACAGGA	*pgaABCD operon* of *E. coli*	Kpn1
VG51r	GTCGATCTAGAGGTGTTTATGCC CGGACT	*pgaABCD operon* of *E. coli*	Xba1
VG52f	ATCGGATATCTTGTAAATATAACC GTCTCCG	*rmf promoter* of *E. coli*	EcoRV
VG52r	ACAGACCATGGGCCTCGTTTCCC TCATAC	*rmf promoter* of *E. coli*	Nco1
VG53f	TAGCGAATTCATGGATGATATTAA TCTATTTCCG	*agrCagrA CDS* of *S. epidermidis*	EcoR1

VG53r	TCAGGGATCCTGGGTGTTTCATTA TATTTTT	*agrCagrA CDS* of *S. epidermidis*	BamH1
VG53rB	CACCAGATTAATCGTATCTACAT TACTTTCTTTTG	*agrCagrA CDS* of *S. epidermidis*, contains overlapping sequence with VG57f	
VG53rT	CGTACAAGGGATCGTATCTACAT TACTTTCTTTTG	*agrCagrA CDS* of *S. epidermidis*, contains overlapping sequence with VG58f	
VG54rb	TGCAGGATCCACTACTCTCCTCA AGTGTCA	*RNAIII promoter* of *S. epidermidis*	BamH1
VG54rx	AGTCCTCGAGACTACTCTCCTCA AGTGTCA	*RNAIII promoter* of *S. epidermidis*	Xho1
VG55f	AGTCCTCGAGGGTTAGCGCCAG ATTTTAAC	*yhjX promoter* of *E. coli*	Xho1
VG55r	TGCAGGATCCAAGGTGTCATGG CAGTATTC	*yhjX promoter* of *E. coli*	BamH1
VG56f	ATCGCTCGAGTATCCATAGTAAA ACCTGGCAT	*yjiY promoter* of *E. coli*	Xho1
VG56r	TCGAGGATCCTGATACGTTTTAA CATGGTTTCT	*yjiY promoter* of *E. coli*	BamH1
VG57f	AATGTAGATACGATTAATCTGGT GAAAGATGAGC	lytTR domain of *ypdB* gene of *E. coli*	
VG57r	TCAGGGATCCTTAAAGATGCATT AACTGGCG	lytTR domain of *ypdB* gene of *E. coli*	BamH1
VG58f	AATGTAGATACGATCCCTTGTAC GGGGCATAG	lytTR domain of *yehT* gene of *E. coli*	
VG58r	TCAGGGATCCTTACAGGCCAAT CGCCTCTTTT	lytTR domain of *yehT* gene of *E. coli*	BamH1
VG70f	GATCCTCGAGTTCATGCTTTTC TCCACCAG	*trxC promoter* of *E. coli*	Xho1
VG70r	GTACGGATCCCTAACCTCGGGA TGAGTAAG	*trxC promoter* of *E. coli*	BamH1
VG71f	GATCCTCGAGATCGCGCGCATA CGCTTCC	*grxA promoter* of *E. coli*	Xho1

VG71r	GATCGGATCCTATTTCTCTCCTC ATAGATTTATGCCTGT	*grxA promoter* of *E. coli*	BamH1
VG72f	GATCCTCGAGCATTATCCATCCT CCATCGC	*oxyS promoter* of *E. coli*	Xho1
VG72r	GATCGGATCCTCCGTTTCTGTG AGCAATTA	*oxyS promoter* of *E. coli*	BamH1
VG73f	GATCCTCGAGAAGCAGAGCCAG TAAAAGTAT	*ahpC promoter* of *E. coli*	Xho1
VG73r	GATCGGATCCTCTATACTTCCTC CGTGTTT	*ahpC promoter* of *E. coli*	BamH1

List of abbreviations

Table 20.5. – List of abbreviations

Abbreviation	Full form
AIP	Auto-inducing peptide
amp	Ampicillin
CDS	Coding sequence
CFP	Cyan fluorescent protein
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FRET	Fluorescence Resonance Energy Transfer
GFP	Green fluorescent protein
IPTG	Isopropyl ß-D-1 thiogalactopyranoside
kan	Kanamycin
LB	Luria-Bertani
MBTH	3-methyl-2-benzothiazolone hydrazone hydrochloride
MCP	Methyl-accepting chemotaxis protein
mRNA	Messenger RNA
NAG	N-acetyl glucosamine
NP-GlcNAc	4-nitrophenyl N-acetyl-ß-D-glucosaminide
OD	Optical density
PEG	Poly ethylene glycol
PGA	Poly N-acetyl glucosamine
PTS	Phosphotransferase system
RBS	Ribosome Binding Site

RNA	Ribonucleic acid
rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide Gel Electrophoresis
TAE	Tris acetate EDTA
TB	Tryptone broth
TCS	Two-component system
TE	Tris-EDTA
TSS	Transformation storage solution
YFP	Yellow fluorescent protein

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