

**A SYNTHETIC BIOLOGY APPROACH
FOR ENGINEERED FUNCTIONAL
BIOFILM**

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A SYNTETHIC BIOLOGY APPROACH FOR ENGINEERED FUNCTIONAL BIOFILMS

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December 2017

We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

A SYNTHETIC BIOLOGY APPROACH FOR ENGINEERED FUNCTIONAL BIOFILM

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PhD in Materials Science and Nanotechnology

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Extracellular polymeric substances consist of molecules, DNAs, carbohydrates, and proteins that are secreted by microbial biofilms. These molecules assist in the synthesis of bacterial biofilms as highly ordered, complex and dynamic material systems, contribute to the adaptation of cells to their environment, and increase their flexibility and functionality under a broad range of conditions. Bacterial biofilms are promising tools for functional applications as bionanomaterials. They are synthesized by well-defined machinery, readily form fiber networks covering large areas, and can be engineered for different functionalities.

One aspect of the present thesis focuses on controlling the expression of the curli proteins of *Escherichia coli* and functionalize the curli fibers by genetically fusing various peptide molecules. Biofilm proteins were functionalized with designed conductive aromatic aminoacids by using programmed cellular machines in order to develop electrically conductive protein nanofiber networks. It has been shown how biological conductivity can be used to control and direct metabolic activities of bacterial populations. Understanding and building conductive biological

interfaces to merge living systems with electronic gadgets is a demanding subject. First time in the literature we succeeded to demonstrate living cells enabled bio-conductivity via a conductive nanofiber network formation.

In *E. coli*, there are two proteins as backbones of the nano-fibers (CsgA and CsgB) responsible for the formation of biofilms. In this thesis, tunability of the morphology and mechanical properties of biofilm backbones were investigated by using protein engineering. The effect of minor and major proteins and their engineered form on the final mechanical properties of the biofilm structures were probed by scanning probe microscopy. The minor protein plays a crucial role in tuning the mechanical and morphological properties of the biofilm structures. Biofilm protein engineering for material science can be used through the genetically tunable biofabrication of self-assembling functional materials.

Using synthetic biological tools, externally controllable biofilm patterns can be achieved. Recombinase based genetic logic gates encoding AND, and OR to control the expression of structural protein CsgA with 6x-His tag modification were engineered with using two independent control signals.

In this thesis, the opportunity to engineer bacterial biofilms using synthetic biology approaches was demonstrated.

Keywords: Biofilm, Synthetic Biology, Curli, Conductivity, Logic Gate

ÖZET

TASARLANMIŞ FONKSİYONEL BİYOFİLMLER İÇİN SENTETİK BİYOLOJİ YAKLAŞIMI

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Hücre dışı polimerik maddeler moleküller, DNA'lar, karbonhidratlar ve mikrobiyal biyofilmler tarafından salınan proteinlerden oluşur. Bu moleküller bakteriyel biyofilmlerin yüksek derecede düzenlenmiş, karmaşık ve dinamik malzeme sistemleri olarak sentezlenmesine yardımcı olur, hücrelerin çevreye uyulanmasına katkıda bulunur ve geniş koşullar aralığında esneklik ve işlevselliklerini artırır. Bakteriyel biyofilmler, biyo-nano malzemeler gibi fonksiyonel uygulamalar için umut vadeden araçlardır. İyi tanımlanmış makinelerle sentezlenirler, geniş alanları kapsayan fiber ağları oluştururlar ve farklı işlevler için tasarlanabilirler.

Mevcut tezin bir yönü, *Escherichia coli* bakterisinin curli proteinlerinin ekspresyonunun kontrol edilmesine ve çeşitli peptid moleküllerini genetik olarak kaynaştırarak curli liflerin işlevselleştirilmesine odaklanmaktadır. Biyofilm proteinleri, elektriği ileten protein nanofiber ağları geliştirmek için programlanmış hücresel makineler kullanılarak tasarlanmış iletken aromatik amino asitlerle işlevselleştirildi. Bakteri popülasyonlarının metabolik aktivitelerini kontrol etmek

ve yönlendirmek için biyolojik iletkenliğin nasıl kullanılacağı gösterilmiştir. Yaşayan sistemleri elektronik aygıtlarla birleştirmek için iletken biyolojik arayüzleri anlamak ve oluşturmak zorunlu bir konudur. Literatürde ilk kez canlı hücrelerin iletken bir nanofiber ağ oluşumu yoluyla biyo-iletkenliği sağladığını göstermeyi başardık.

E. coli'de, biyofilmlerin oluşumundan sorumlu nano-liflerin (CsgA ve CsgB) omurgaları olarak iki protein bulunur. Bu tezde biyofilm yapılarının morfolojisinin ve mekanik özelliklerinin ayarlanabilirliği protein mühendisliği kullanılarak araştırılmıştır. Biyofilm yapılarının nihai mekanik özellikleri üzerine minör ve majör proteinlerin ve bunların mühendislik formunun etkisi, tarama probu mikroskopisi ile araştırıldı. Minör protein, biyofilm yapılarının mekanik ve morfolojik özelliklerinin ayarlanmasında hayati bir rol oynar. Malzeme bilimi için biyofilm protein mühendisliği, kendi kendini monte eden işlevsel materyallerin genetik olarak ayarlanabilir biyo-fabrikasyonu ile kullanılabilir.

Sentetik biyolojik araçlar kullanarak, harici olarak kontrol edilebilir biyofilm kalıpları elde edilebilir. 6x-Histaq modifikasyonlu yapısal protein CsgA'nın ekspresyonunu kontrol etmek için AND ve OR kodlayan rekombinaz esaslı genetik mantık kapıları, iki bağımsız kontrol sinyali kullanılarak tasarlandı.

Bu tezde, sentetik biyoloji yaklaşımlarını kullanarak bakteriyel biyofilmleri mühendisliği yapma fırsatı gösterildi.

Anahtar kelimeler: Biyofilm, Sentetik Biyoloji, Curli, İletkenlik, Mantık Devresi

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CHAPTER 1

Introduction

1.1. History of Amyloids

Amyloid refers to inherited and abnormal fibrillar proteins, which are deposited in organs and tissues, cause inflammatory diseases known as the amyloidosis including Alzheimer's, the spongiform encephalopathies and type II diabetes [1]. The term 'amyloid' was firstly used by Schleiden (German botanist, 1804-1881). He uses the term "amyloid" for starch, applying to "starch-like" in his book "Principles of Scientific Botany" [2, 3]. The term comes from latin word "amylum" for starch.

Iodine stained extracellular, fibrillar amyloid macroscopic tissue deposits related with human disease were first described by Virchow in 1854 [4]. Macroscopic abnormality was thought as cellulose due to blue staining with addition of iodine, and violet after treatment of sulfuric acid until 1859, Friedreich and Kekule draw attention to amyloids as proteins by showing the presence of high nitrogen level

and the absence of carbohydrate in amyloid “mass” [4]. Further, Light microscopy and histopathologic dyes such as thioflavin and Congo red were helped to solve the amyloid structures as they became available to scientist in 1920s [1]. A protein to be considered as an amyloid, although first criterion was the production of apple green birefringence under polarizing filter using light microscopy upon the specific binding of amyloid to it (Figure 1.1), the birefringence was showed by Divry and Florkin (1927) [5] and the color was showed by Ladewing (1945) [6], mixture of colors (yellow-green or yellow, and blue-green or blue) can be observed depending on birefringence properties (relative orientations and strength) of the strain in lenses and the Congo red [7]. Then this finding led Cohen (1959) examined the human primary and secondary amyloid tissues under the electron microscopy showed that amyloids are in the form of fibrillar structure which was the second criterion to be defined as amyloid [8]. Isolation of amyloid fibrils from tissues by physical separation methods showed that amyloids are insoluble in physiologic saline (1968) [9]. As the third characteristic of amyloid fibers are the cross beta structure (Figure 1.2) in which beta sheets are perpendicular to fibril axis which can be analyzed from X-ray diffraction pattern of fibers(1968)(Figure 1.2) [10].

1.2. Structure of Amyloid Fibers

Methods used for protein crystallization are generally inapplicable to amyloid fibers, which are insoluble and therefore difficult to crystallize through thin film evaporation. Other characterization methods, such as Fourier transmission infrared resonance (FTIR), nuclear magnetic resonance (NMR), atomic force microscopy, electron microscopy and X-ray diffraction [11-15], are therefore

preferable for the structural analysis of amyloids such as curli. Electron microscopy images of an amyloid fiber gives the structural morphology of fibers (Figure 1.2.A) and X-ray diffraction analysis reveals the pattern of amyloid parallel β -sheets (Figure 1.2.C)[16].

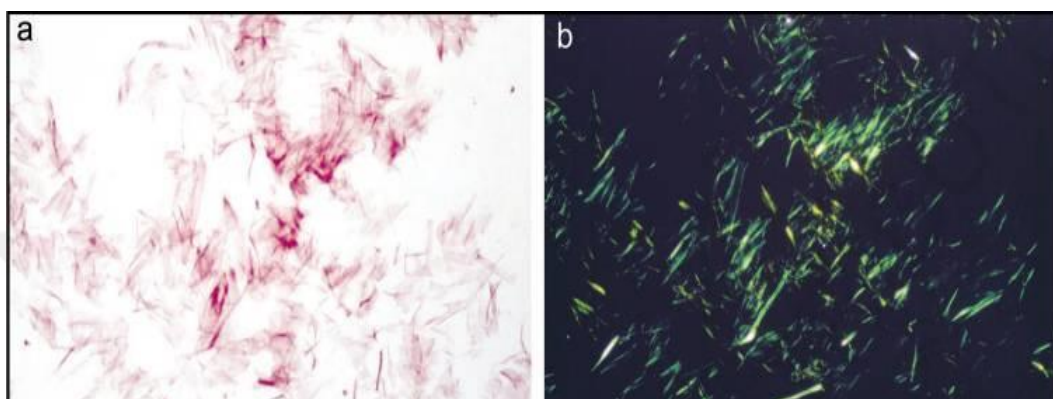


Figure 1.1: Congo red stained isolated amyloid fibers of fragment of fibrinogen under light microscopy (a) and between polarizing filter showing the characteristic green apple birefringence of amyloid fibers (b). Figure adapted with permission from reference [17].

Secondary structure of amyloids are mainly consist of β strands, and α helices, turn, loop, and bends are found in the core structure [18-20]. Although the cross β -sheet structure of β -amyloid are suggested to be an anti-parallel β -sheets in early study, later on it was showed that the secondary structure of amyloids are composed of parallel beta sheets[21-23]. Paralel β -sheets are rarely found in protein structures due to low stability (less energetic) [24]. Interactions between β -sheets and orientation of β -sheets contribute to amyloid formation, ordered sturucture and stabilize the structure [25]. Interactions can be intermolecular or intramolecular depending on single molecule layer or multiple layers[26]. Hydrogen bonding between parallel β -sheets is the common structure between

fibrous molecules (Figure 1.2.B)[1]. X-ray crystallography studies of short amyloidogenic peptides has showed their atomic structure which are consist of variable “steric zippers” [27, 28]. Small angle X-ray scattering and cryo electron microscopy studies showed that fibrils are composed of several protofilaments or monomers [29-32].

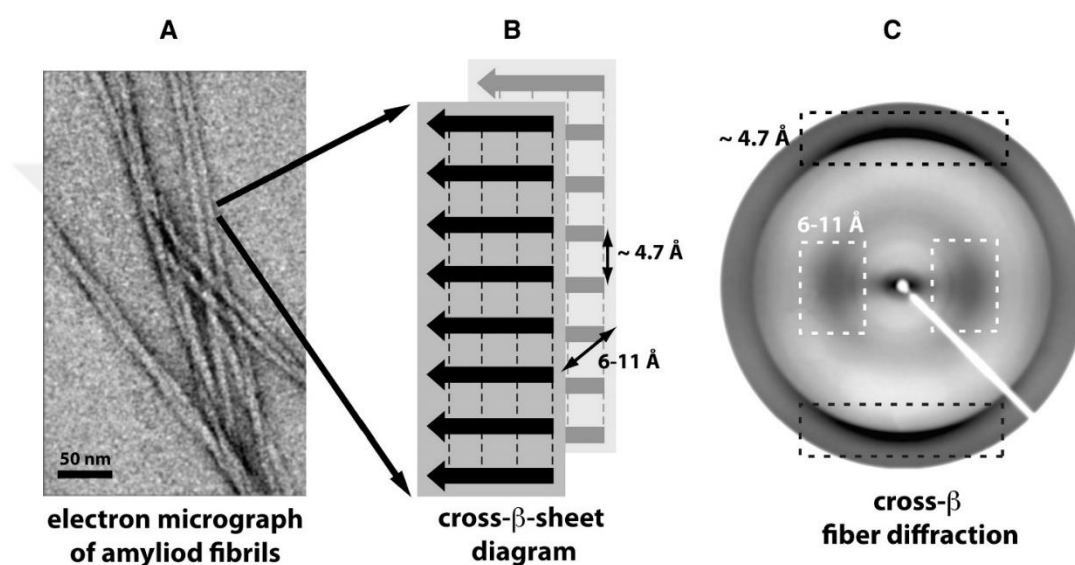


Figure 1.2: Structural analysis of amyloid fibers. (A) Electron microscopy images of Val30Met transthyretin amyloid fibrils showing long, straight and un-branching fibrils[33]. (B) Schematic diagram of the cross-β-sheet in a fibril, the dashed lines shows the hydrogen bonding between β-sheets. (C) X-ray diffraction pattern of amyloid fibers with black dashed box on the meridian reflecting at 4.7 Å and white dashed box on the equator reflecting at 10 Å. Reprinted with permission from reference [16].

1.3. Functional Amyloid Fibers

Previously written in the text, amyloids are related with many diseases and thought to be non-functional. In many cases, amyloids were found as functional

part/state of proteins in nature according to recent studies (Table 1). As mentioned before, their structural similarities were also cross- β structure and they are regulated strictly by a special machinery. Many species such as enteric bacteria, fungi, yeast, and also human, synthesize functional amyloids (Table 1). They have a role in cellular activities such as interaction with host tissues, maintaining homeostasis of the organism, remodeling of the extracellular matrix, biofilm formation, and evasion of immune system.

Species	Protein
Human	PmeI17[33]
Insects and fish	Chorion proteins[34-36]
<i>Nephila clavipes</i>	Spidroins[37]
Macroalgae	Sawtooth[38]
Microalgae	Sawtooth[39]
Fungi	Hydrophobins[40-44]
<i>Podospora anserine</i>	HET-s[45-48]
<i>Saccharomyces cerevisiae</i>	Swilp[49],Sup35[45, 49-51],URE2P[45, 49, 52]
<i>Escherichia coli</i> , <i>Salmonella enteritidis</i>	Curli[53] [43, 53-56]
<i>Pseudomonas sp.</i>	FapC[57]
<i>Streptomyces coelicolor</i>	Chaplins[43, 58, 59]
<i>Bacillus subtilis</i>	TasA[60]
<i>Klebsiella pneumoniae</i>	Microcin E492[61]
<i>Staphylococcus aureus</i>	PSM[62]
<i>Streptococcus mutans</i>	Adhesin P1[63]
<i>Mycobacterium tuberculosis</i>	MTP[64]
<i>Xanthomonas axonopodis</i>	Harpin[65, 66]

Table 1.1: Functional amyloids found on species.

Mammalian protein Pme17 which has amyloidogenic properties and fibrous structure is located on melanosome and functions in melanin synthesis [30]. In addition, hydrophobins which are also amyloid like proteins take role in fungal development and form a water repellent coat during sporulation [34]. Moreover, although the polymeric form of recombinant mammalian prion protein (rPrP),

which has β -strands in its structure induces cell apoptosis for cultured cells, monomeric form of rPrP, which has α -helices in its structure induces formation and development of neurite [35].

Furthermore, *fab* operon which encodes six proteins (FabA-F) was discovered in biofilm forming bacteria *Pseudomonas* [36]. It increases the biofilm formation capacity in *Escherichia coli* by overexpressing [36]. FabC is the structural amyloid component of *fab* operon [37].

Opportunistic pathogen bacteria *Staphylococcus aureus* produces Bap proteins, which are biofilm associated proteins [38]. Bap proteins formation are induced to build biofilm matrix by environmental stimuli such as low pH and low calcium concentration [38]. Cation molecules prevent the formation of amyloid fibers by binding to the molten globule like states of Bap proteins [38]. Bap proteins function as environmental sensor and scaffold protein for biofilm formation [38]. They are composed of repetitive structure, high molecular weight proteins and are found at the cell surface [39]. Initial attachment to different surfaces and interaction with bacterial cells were developed by Bap proteins [39]. Pathogen escaped from immune cells using Bap proteins by preventing internal localization [40, 41]. Bap proteins bind to host receptors [39]. There are also phenol soluble modulins (PSMs) which are small peptides take role in biofilm integrity in *S. aureus* species [42]. PSM mutants lack robust biofilm structure and biofilm structures of mutants were easily disrupted by mechanical stress and enzymes [42]. PSMs are found as aggregated fibrils in the biofilm matrix and function in metabolic activities [42]. It was showed that they have antimicrobial activity against other bacteria [42]. They escaped from host immune responses by

recruiting and lysing neutrophils [42]. In addition, Methicillin-resistant *Staphylococcus aureus* (MRSA) strains synthesize cytolysin (PSM) which is related with virulence factors [43].

Mycobacterium tuberculosis also produces special type of pili called MTP (*M. tuberculosis* pili) which are formed by functional amyloid proteins [44]. MTP has binding ability to the laminin which is an extracellular matrix protein shows importance for colonization of bacteria [44]. They share common morphological, functional and biochemical properties with curli amyloids [44].

Finally, Endospore forming *Bacillus subtilis* forms a coat by complex proteins which resist against stress factors protease treatment, pH changes, etc. Main components of the matrix is Exopolysaccharides and TasA which is related with spores and antimicrobial activities [45]. TasA were proves as the major structural component of amyloid fibers and critical for biofilm integrity [45, 46]. Endospore formation is synergistically controlled by TasA and GerE [81]. Another protein SipW which has bifunctional activity: signal peptidase and regulatory roles also controls the maturation of TasA fibers [47]. Self-assembled TasA fibers has similar properties with functional amyloids [48]. Nucleation of TasA monomers is assistant by TapA protein [48]. It was showed that TasA fibril formation is required 8 amino acid residues at the N terminal of TapA [48].

1.4. Biofilms: Advantages of living together

Microorganisms switch between two stages of growth (Figure 1.3): planktonic phase and sessile phase, that depends on internal dynamics of the system and environmental effects. In biofilm, sessile cells have an increased interaction and

coordination with the environment on the surface while favoring the proliferation and protecting the bulky center whereas in planktonic form, single cells can spread over and colonize new environments which increasing communal longevity [49]. In their life cycles, they are mainly found as sessile state in biofilm [50-52]. Contrary to planktonic state, they are dormant and resistant to environmental threats in biofilm. In natural environments, biofilm structure is composed of diverse microorganisms having diverse ecological interactions (e.g., commensalism, mutualism, competition, predation, or parasitism) [53-57].

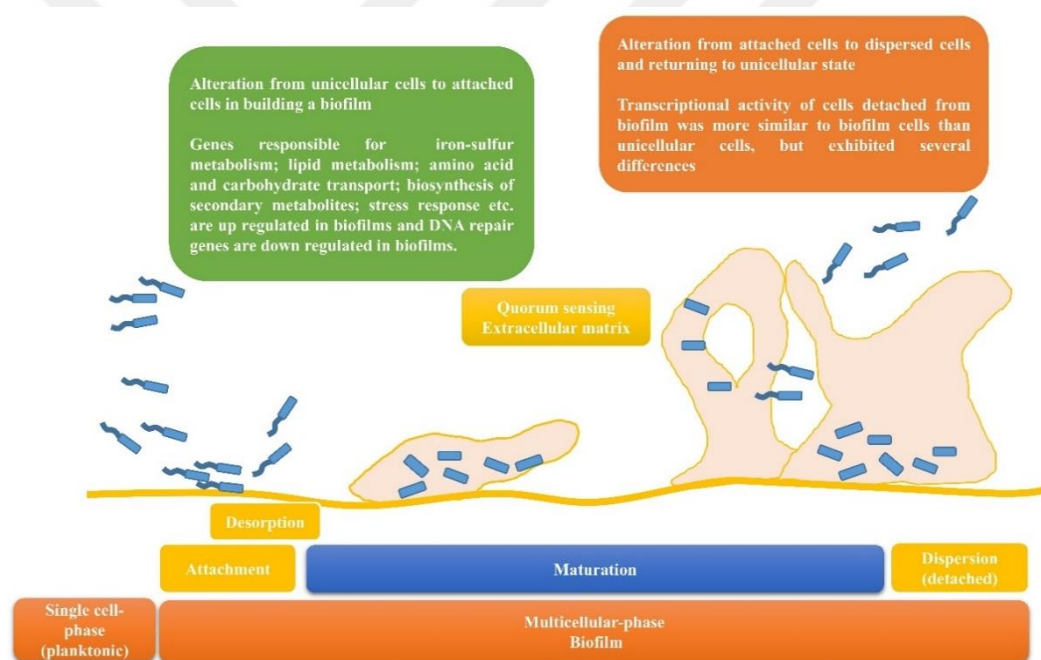


Figure 1.3: The Bacterial Life Cycle. Multicellular cells detach from biofilm using either passive forms (desorption and detachment in the presence of physical force, erosion and sloughing) or active forms (detachment in a controlled manner through changes in the physiological conditions of cells) of dispersal [58]. In both stages of the life cycle, cells undergo transcriptional and gene expression changes.

Biofilm formation among microorganisms shares common features which can be divided into three distinguished stages: attachment, maturation and dispersion (Figure 1.3) [59, 60]. Factors affecting the initial attachment are surface free energy, roughness, hydrophobicity, and electrostatic charges [61].

Biofilms are consist of extracellular matrix for over 90% and microorganisms for less than 10%. Microorganisms secrete many biological substances consisting diverse biopolymers called extracellular polymeric substances (EPS) which build up the three dimensional architecture of the biofilm. EPSs help biofilm cells to adhesion to surfaces and cohesion each other and give many advantages for biofilm cells.

EPSs keep biofilm cell intact form allowing them interact with each other including cell-cell communication which depends on small diffusible auto inducers (quorum sensing molecules) such as acyl-homoserine lactones, and oligopeptides [62] and form synergistic interactions between different types of microorganisms [63]. Extracellular enzymes, preserved in matrix, such as hydrolytic enzymes digest the nutrients from water phase and allow them utilize as energy sources [64]. Biofilm matrix also protect cells from desiccation by retention of water and facilitate horizontal gene transfer among biofilm cells [54, 65-68].

During infection, the protective layer of biofilm provides a barrier against specific and non-specific host defenses such as antimicrobial agents [69], protecting nitrogenase enzyme of cyanobacteria from noxious effects of oxygen [70], and reducing grazing of protozoa on bacteria [54, 71, 72].

1.5. Biotechnological Applications of Biofilms

Biofilm cells secrete some of the compounds which may be used for biotechnological applications, cosmetics, food, and pharmaceutical industries. Amyloid fibers, generally considered as pathogenic factor, in microorganisms have several biotechnological utilization as either bio-templates or functional nanofibers [73, 74].

Microbial surfactants or biosurfactants produced by variety of microorganisms as surface active metabolites[75]. Microbial surfactants act as chemical surfactants that reduce the surface tension and interfacial tensions and microbial versions come with beneficial effects: low toxicity, high biodegradability, environmental fitting, better foaming, and stability at high pH, salinity and temperature[76]. They are used in many industrial process such as agents for emulsion, wetting, foaming, phase dispersion[76]. They have also antibacterial, antifungal and antiviral properties [77].

Another example of commercial protein in biofilm is BslA which is the family member of hydrophobin proteins. It is produced by *Basillus subtilis* on the surface of agar plates or in floating biofilms (pellicles) at the air/liquid interface[78]. Their structure has extremely hydrophobic “cap” region which allows non-wetting biofilm to form at different interfaces and against up to 80% ethanol, other organic solvents, and commercial biocides[79]. They can be used as food stabilizer, or emulsifier in cosmetic products. BslA can be used in ice-cream production which makes more stable in hot weather, keeps more smooth texture, and allows less saturated fats[80].

1.6. History of Curli

Curli proposed as the third surface organelle beside flagella and pili by Stafan Normark in 1989 while investigating *E.coli* isolates from Bovine mastitis for the ability to attach to extracellular matrix glycoprotein fibronectin of eukaryotic cell [81]. In their study, half of the isolates showed the ability to bind fibronectin when they were grown on colonization factor antigen (CFA) agar under conditions such as low temperature (26 °C) that favors curli production [81]. The electron microscopy images of the structures showed tiny, wiry fibres with a diameter of ~2nm that they termed as 'curli' [81]. Curli fibers were also discovered on another enterobacteria: *Salmonella enteredis* by Collinson *et al.* [82]. They named the fibers as thin aggregative fimbriae or Tafi, and proposed them as separate class of fimbriae and not related with curli of *E.coli* [82] because of Tafi fibers production at 37 °C on liquid media [82]. Nonetheless, amino terminal sequencing of curli, csgA, by sequential Edman degradation showed 85% homologous to Tafi and they belong to same class of protein [83].

1.7. Components of Curli Proteins in *E.coli*

Polymerization of curli fibers were firstly described as extracellular self-assembly mechanism of soluble monomers requiring a specific nucleator protein [84-86]. Curli monomers secreted by type VIII secretion system (the extracellular nucleation- precipitation pathway) [87]. Expression and secretion of curli fiber units or Congo red binding curli fibers are controlled by two divergently transcribed operons, *csgDEFG* and *csgBAC*, in *E.coli* (Figure 1.4) [88]. The operon *csgBAC* encodes major subunit CsgA and minor subunit CsgB which are

structural curli subunits. CsgB has %30 sequence homology with CsgA. They are both secreted to extracellular space. CsgB is localized on cell membrane and deletion of 19 amino acids from its C-terminal sequence of CsgB prevent the association with cell membrane [89]. It has been showed that truncated form of CsgB is required for the polymerization of soluble CsgA [89]. Approximately %30 of the wild type *E.coli* cells produces curli fibers on YESCA agar at 26 °C for 48h [90]. ‘Interbacterial complementation’ showed that csgB mutant (A⁺B⁻) cells secrete CsgA monomers can polymerized on the surface of CsgA mutant (A⁻B⁺, representing csgB on cell surface) [85, 86]. Cross-seeding of bacterial amyloids between *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Citrobacter* lead the polymerization of subunits into curli fibers *in vitro* [91]. Furthermore, *E. coli* and *Salmonella enterica* serovar Typhimurium curli mutants were able to cross-seed the curli subunits *in vivo* and restores the ability of bacterial adherence to agar surfaces [91]. The selective inhibitor CsgC prevents soluble curli subunits into insoluble fibers in the periplasmic space [92] and apparently by inhiting the first stages of amyloid formation [93]. Recently, *in situ* study showed that CsgC binds to termini of curli fiber during elongation and decreases the growth rate and elongation length [94].

The operon *csgDEFG* encodes CsgD regulator which controls the transcription of *csgBA* promoter and three curli export machinery that is required for the transportation of curli subunits and polymerization into fibers [88]. CsgG forms nanomeric ring-shaped pore in the outer membrane which facilitates secretion of curli subunits [90, 95-97]. Immunoprecipitation of CsgG with either CsgE or CsgF showed the existence of interaction between them at the outer membrane

[96]. CsgE and CsgF is thought to act as a chaperon-like protein in the assembly of structural curli proteins [86]. Both sequence analysis based on charge and hydrophobicity and secondary structure analysis with circular dichroism spectroscopy of CsgE and CsgF proteins showed that they have disordered regions as similar some chaperon-like proteins [98]. Periplasmic protein CsgE inhibits the passage of non-specific proteins through CsgG pore and the self-assembly of CsgA into fiber in the periplasma [99].

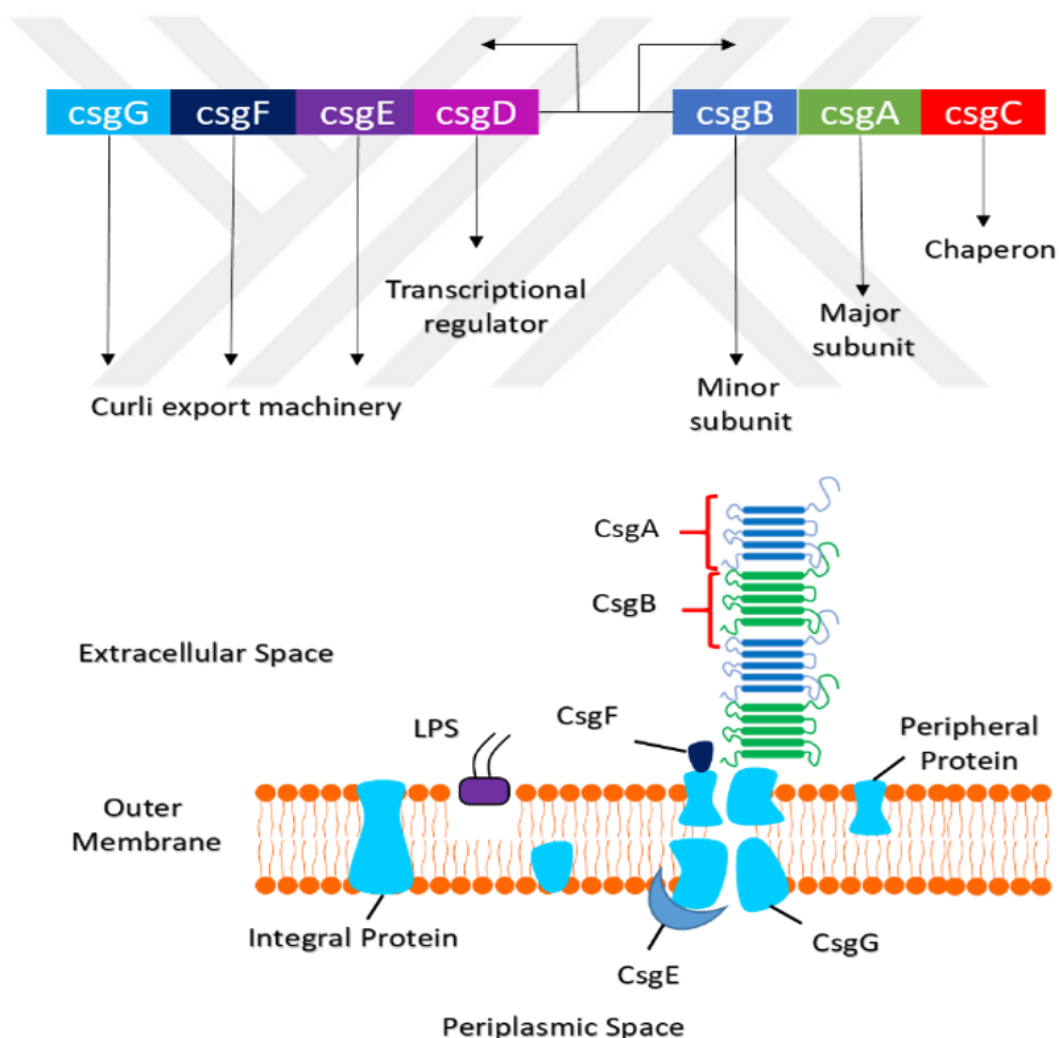


Figure 1.4: Curli proteins involves in biofilm production. The *curli* operon includes CsgB that helps CsgA to polymerase on top of each other. The second operon (*csgDEFG* operon) controls the regulation of gene expression and

anchoring, forming pores and transformation the CsgA to the surface. *Curli* proteins pass through Sec pathway to function.

CsgE forms a complex with CsgG by sealing the periplasmic space of CsgG which entraps CsgA allowing unidirectional across the outer membrane by entropic potential over channel [95]. CsgA polymerization at different stages (0h, 1h, 7h, and 8h) was inhibited and CsgA fibers were arrested by CsgE addition. CsgE also found to inhibit pellicle formation by addition of purified CsgE into liquid media. In addition to, CsgA fiber formation and cell membrane association of it are both required CsgF function in vivo. Finally, CsgF is secreted into the media and mediates surface localization of and/or chaperoning of CsgB for efficient nucleation.

1.8. Engineered Functional Curli Fibers

Functional amyloids generated by fusing functional peptides or proteins leads to development of bio-nanomaterials [100, 101]. Designed materials are secreted to the extracellular matrix by its secretion machinery such as outer-membrane-localized CsgG pore in *E.coli* [96]. Secreted fusion protein self-assembles into nanofiber network with multifunctional properties under the control of inducer molecules in a programmable manner. Adjusting the concentration of inducer molecule, it is likely to obtain precise multiple designed functions which were previously studied with functional proteins, peptides or molecules that binds to surfaces or to materials [102, 103], act as a template for the control of nanoparticle formation [104-106], allow supra-molecules to attach via covalent or non-covalent affinity tags [107-109], show catalytic activity found in bacteria

[110], allow electron transfer [111], or allow light harvesting [112]. Bio-inspired hybrid fibers or bio-catalytic surfaces can also be designed by fusing whole proteins to curli fibers [113, 114]. Fusing the peptide domains to CsgA protein with SpyTag/SpyCatcher has been also demonstrated that many functions such as binding to metals or minerals, templating nanoparticles or optically active quantum dots, biomineralization of bone, and a tag for covalent bond formation to larger proteins [115]. Another system using affinity tags for gold nanoparticles and quantum dots to create organic-inorganic interfaces reflect electrically conductive and optically active biofilms [116, 117].

Engineered 'living functional materials' obtained from bacteria by using synthetic tools can be utilized for various applications (Figure 1.5). Biofilms are used to produce various fermentation products (ethanol, butanol, lactic acid, and succinic acid) or for waste water treatment or environmental remediation. By an appropriate reactor design and suitable solid support for engineered biofilms increases the production of the desired product and allows the equal distribution of the biofilm [118].

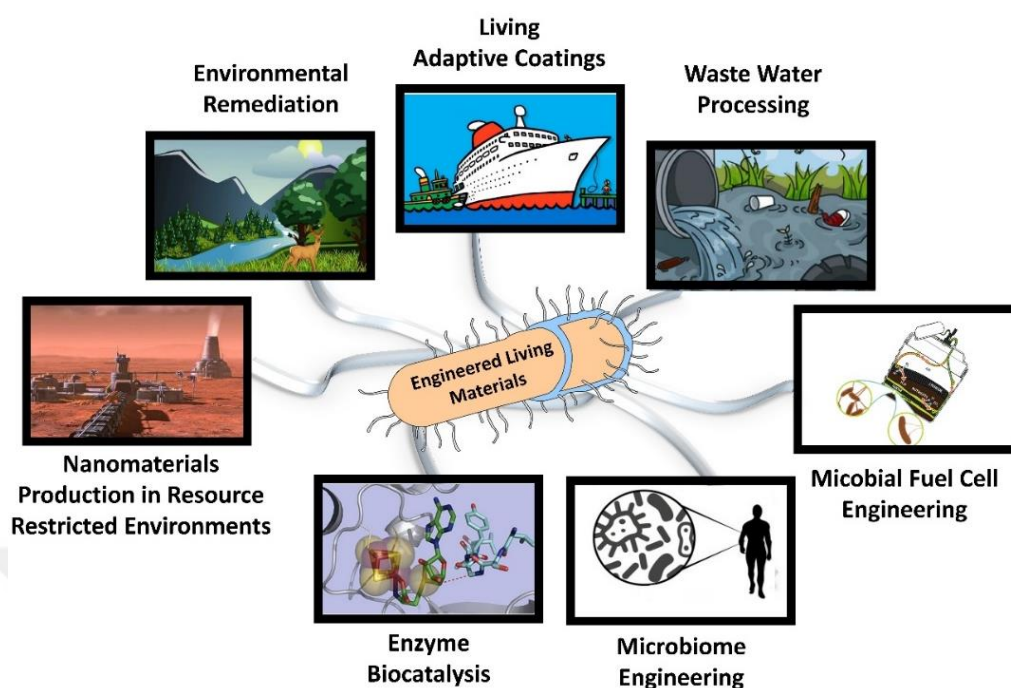


Figure 1.5: Potential Applications of Engineered Living Materials.

One could use the strategy of ureolytic organisms, which immobilize the toxic metals in soil by precipitation [119], with the combination of engineered biofilm as cell based and various nanomaterials in environment remediation [120]. Waste water treatment processes includes the activated sludge system, up-flow anaerobic sludge blanket (UASB) system, and partial nitrification/anaerobic ammonium oxidation (Anammox) system [121]. In all systems, naturally occurring bacteria such as heterotrophic bacteria for organic carbon consumption and removal, coexisting bacteria for nitrogen removal, denitrifying bacteria for anaerobic ammonium oxidation ('Anammox') [122]. Biofilm engineering could enhance the ability of the waste water treatment of naturally occurring bacteria with desired properties required for a specific mainstream.

Another application of engineered biofilms is the microbial fuel cell (MFC) technology that is the production of electricity by microbially catalyzed organic

waste in anode. Mechanisms of electron transfer between bacteria and the electrode, and surface chemistry and surface morphology of electrode are critical for optimal fuel cell efficiency [123, 124]. Electron transfer mechanism could be direct transfer via conductive pili [125] or cytochrome proteins [126] or indirect transfer via mediators or fermentation products [127]. Therefore; it is important to engineer adhesive, physical or mechanical properties of the biofilm nanomaterials to adapt for improvements in MFC technology.

Combination of the constructed wetlands (CW) and microbial fuel cells (MFC) can also be used for waste water treatment and electricity generation [128]. CW-MFCs requires redox gradient which anaerobic bacteria for waste water treatment present at anode and aerobic bacteria present at cathode and therefore biofilm engineering could be beneficial to combine two systems.

Artificial biofilms could be used as protective layer for biomaterial or non-biomaterial in the environment due to aging problems of all materials. Biofilms could be engineered to adhere to specific material surfaces even giving a new property such as anti-corrosion and microbial antagonistic mechanism (biocontrol agent) [129, 130]. Anti-corrosion can be achieved by forming barriers against corrosion products such as aluminum surface produced by *Bacillus licheniformis*, consumption of oxygen with aerobic bacteria, secreting corrosion inhibitors such as siderophores, or antimicrobial action against corrosion-causing organisms such as sulfate reducing bacteria [130, 131].

Genetic circuits which sense chemical compound and toxic compound combined with signal output in the form of light such as fluorescence, luminescence, or a

colorimetric output from enzyme activity by bacterial bioreporters has been intensively studied for long times [132]. Engineered biofilm materials could be combined with genetic circuits to increase stability and survivability of whole cell biosensor platforms in the harsh conditions of environment. Even genetic circuit output could be link to electronic circuit with conductive biofilm nanofibers.

One of the grand challenges of space synthetic biology is *in situ* resource utilization (ISRU) which aims to reduce the expensive transport of equipment and consumables from the Earth into space. In order to start ISRU in space, microbiomes should adapt to extreme environments fluctuations such as temperature, ionizing radiation, and minimal nutrient and oxygen availability [133]. Biofilm communities has already achieved to adapt extreme environmental conditions of earth in the way of evolution and creating engineered biofilm materials could help suitable environment by changing the properties and dynamics of such microbes. Engineered biofilms could be used to process the space resources such as solid waste, volatiles, and geological materials on site. As previously mentioned, light harvesting strategies combination with biofilm materials could be used to produce oxygen, fuel and food from available carbon dioxide, sunlight, water on Mars [134].

CHAPTER 2

Cloning and Expression of Functional Curli Fibers

2.1. Introduction

Escherichia coli produces functional curli fibers that are important in early attachment for biofilm formation. Biofilm proteins also contribute to the stiffness of the biofilm matrix. CsgA and CsgB proteins are the structural subunits that are responsible for attachment to abiotic or biotic surfaces. The *csgDEFG* operon contains the regulatory elements that modulate the synthesis of structural proteins and the secretory machinery required for their extrusion to the extracellular environment. In order to control the expression and secretion of the curli protein complex, *csgA*, *csgB*, *csgBA* and *csgEFG* genes were cloned into pZ vectors under the control of P_{LtetO} with riboswitch or $P_{Llac/ara}$.

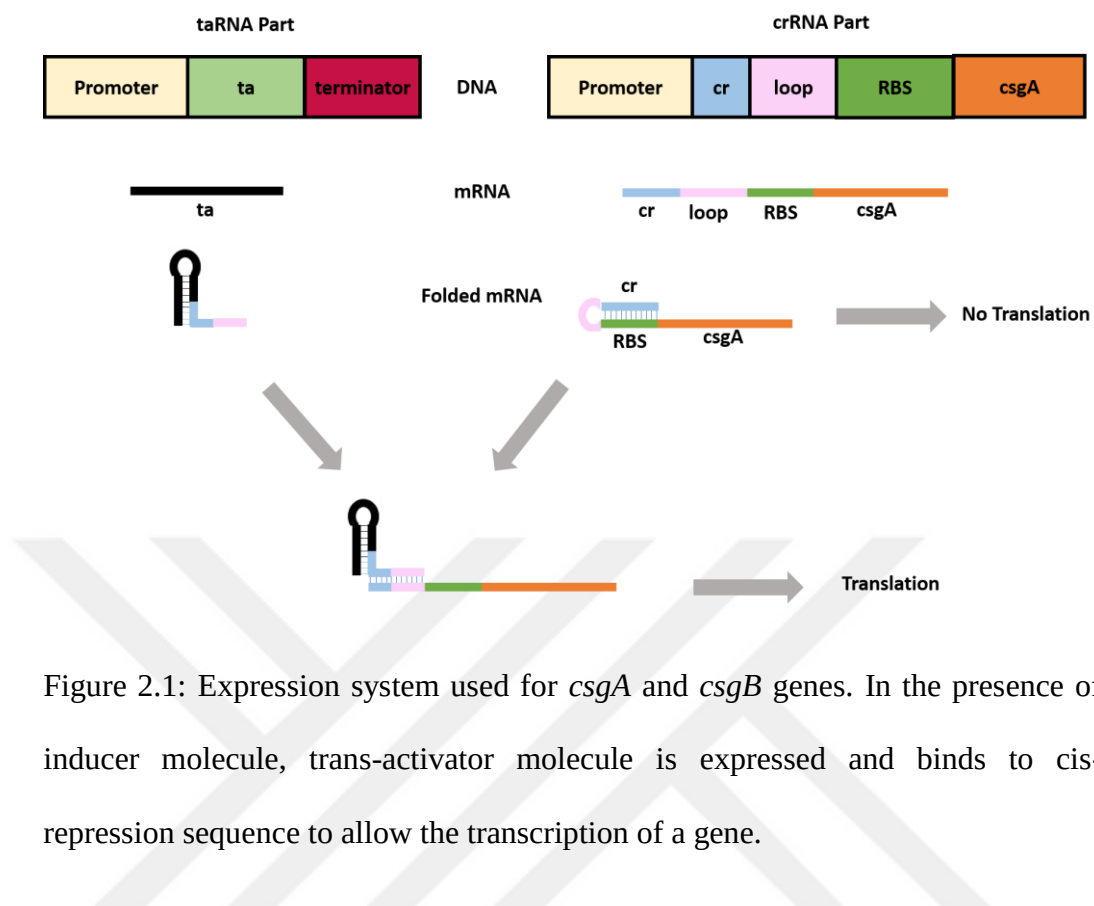


Figure 2.1: Expression system used for *csgA* and *csgB* genes. In the presence of inducer molecule, trans-activator molecule is expressed and binds to cis-repression sequence to allow the transcription of a gene.

2.2. Objective of the Study

Our aim was to obtain the *E.coli* curli expression system in a controlled manner and independent from its genome. We also wanted to characterize the curli fibers produced from exogenous gene circuits. Curli operon genes were controlled under the inducible expression system (Figure 2.2). For this work, major and minor curli subunits deleted strain of *E.coli* and *E.coli DH5α PRO* was used. The system is expected to synthesize the curli fibers over the cell surface.

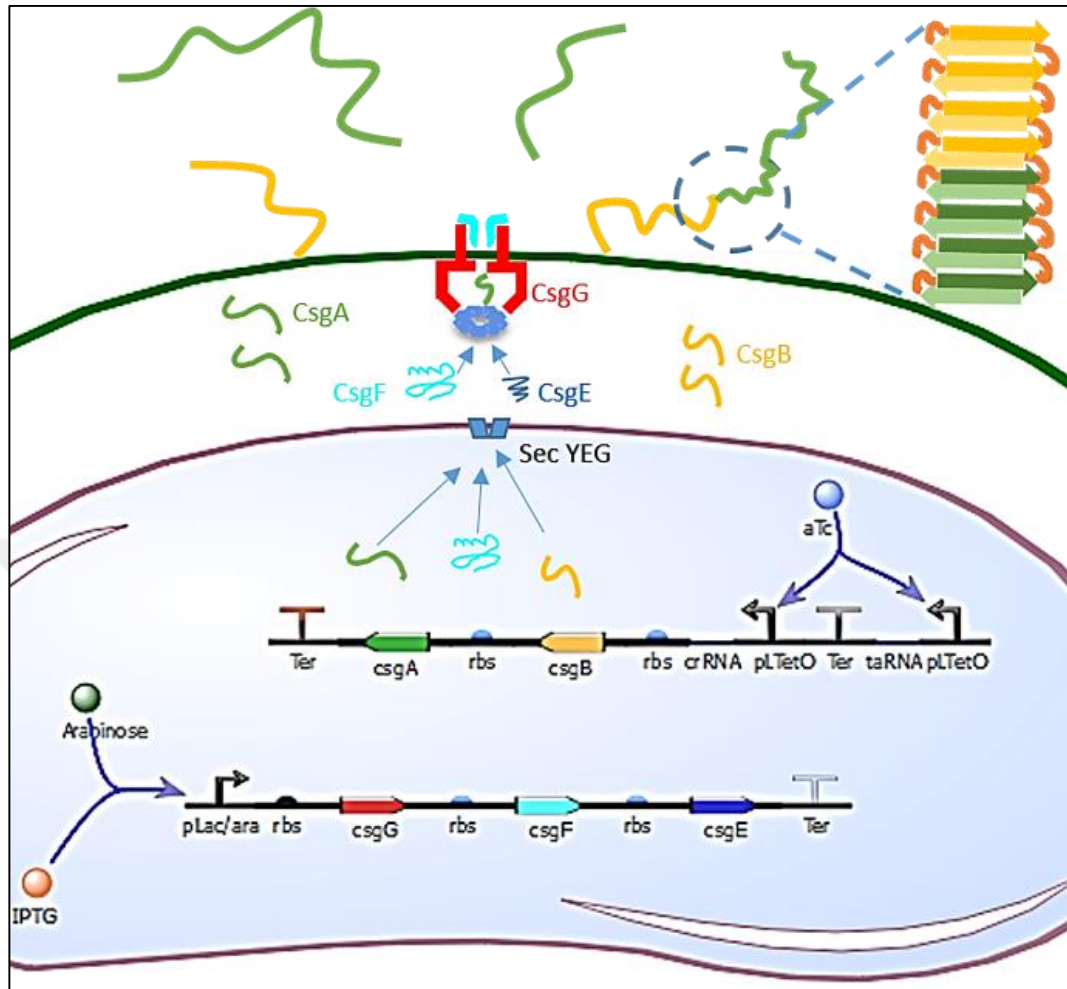


Figure 2.2: Curli operon genes under inducible system and secretion system of the curli fibers.

2.3. Results

2.3.1. Cloning of the *csgA* and *csgB* Genes

In order to obtain curli producing strains, *csgA* gene and *csgB* gene was amplified from pZa-CmR-tetO-ribo-csgBAEFG vector (gift from Prof. Timothy K. Lu) with PCR. Amplified products were visualized on %1 (w/v) agarose gel and gel images are shown Figure 2.3 and Figure 2.4. PCR products were purified from agarose gel using gel extraction kit. PCR products were cloned into pZa-tetO-ribo-CmR

and pZa-tetO-ribo-AmR vectors with cut ligate method. PCR products and vectors were digested with KpnI and MluI endonucleases. PCR products and linearized products were visualized on agarose gel that is shown in Figure 2.5. Plasmid constructs are shown in Figure C.1 and Figure C.2. Sequences were verified by sequence alignment shown in Figure D.1 and Figure D.2.

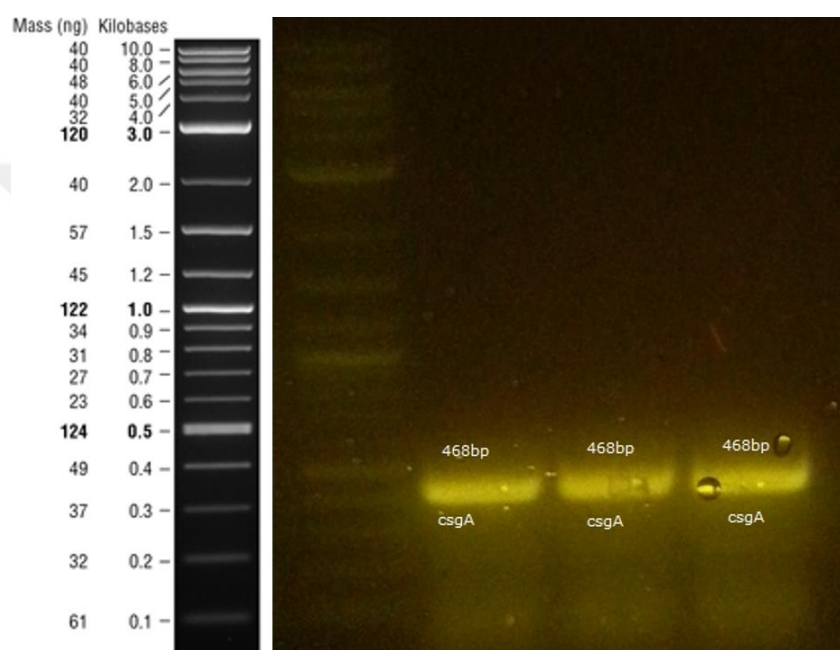


Figure 2.3: *csgA* gene fragments are observed between 500bp and 400bp as expected.

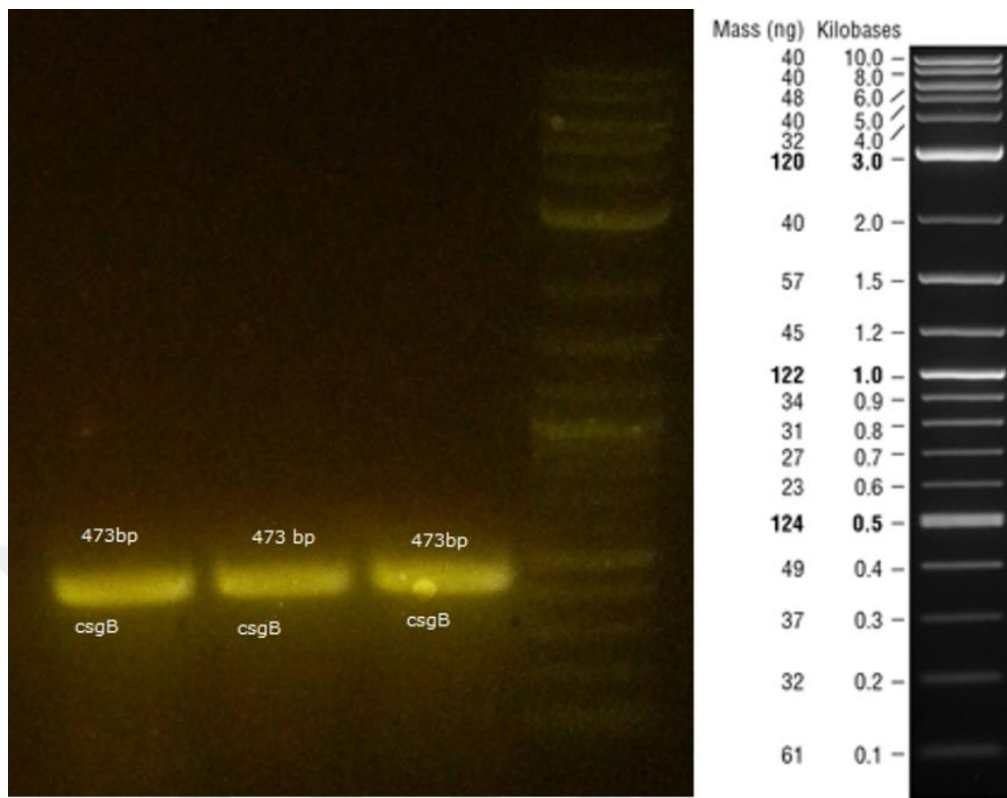


Figure 2.4: *csgA* gene fragments are observed between 500bp and 400bp as expected.

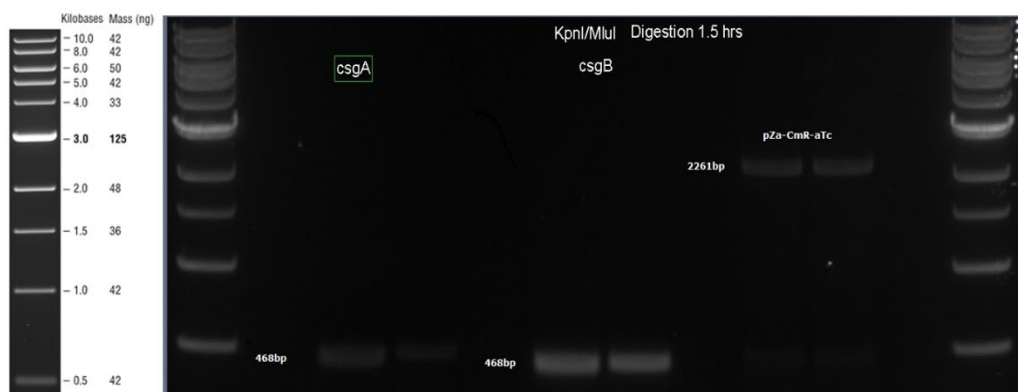


Figure 2.5: Linearized *csgA*, *csgB* were visualized at ~500bp as expected and linearized pZa-tetO-ribo-CmR were visualized at ~ 2200bp as expected.

2.3.2. Cloning of *csgBA* Operon

Further, *csgBA* operon was cloned into pZa-tetO-ribo-CmR vector using cut ligate method. *csgBA* operon was amplified from pZa-CmR-tetO-ribo-*csgBAEFG* vector with PCR using *csgBA* forward and reverse primers (Table B.1). Amplified products were visualized on %1 (w/v) agarose gel and gel images are shown Figure 2.7. PCR products were purified from agarose gel using gel extraction kit. PCR products were cloned into pZa-tetO-ribo-CmR vector with cut ligate method. PCR products and vectors were digested with KpnI and MluI endonucleases. Then, plasmid constructs were analyzed with agarose gel electrophoresis after KpnI/MluI digestion (Figure 2.8) and verified with sequence alignment (Figure D.3). Plasmid construct is shown in Figure C.3.

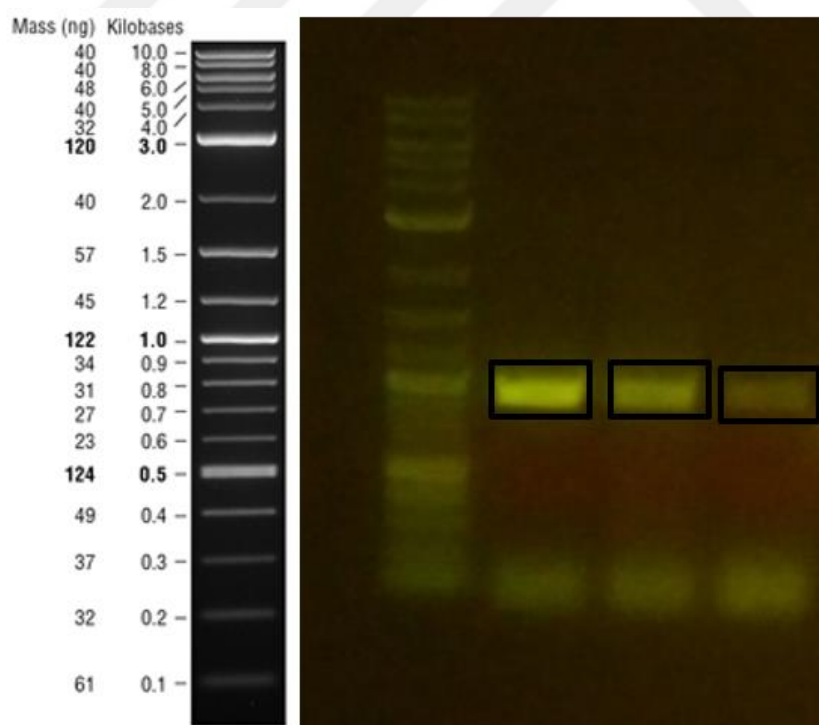


Figure 2.7: PCR amplified *csgBA* gene fragments are observed at ~1kb as expected.

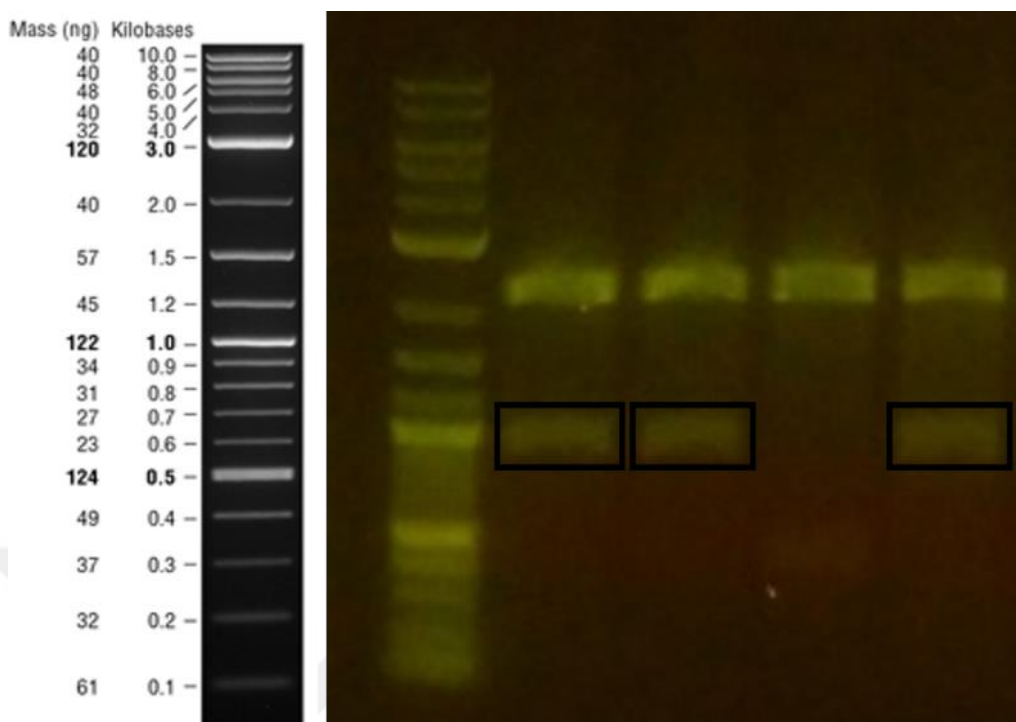


Figure 2.8: pZa-tetO-ribo-csgBA-CmR plasmid constructs were digested with KpnI/MluI to verify *csgBA* genes. *CsgBA* genes were obtained at ~ 1kb in 3 colonies.

2.3.3. Cloning of *csgEFG* Operon

CsgE and *CsgF* genes are cloned into pZs CsgG pLac/ara Kan vector (Figure C.4) with Gibson assembly method. *csgE* and *csgF* genes were amplified from pZa-CmR-tetO-ribo-csgBAEFG vector with PCR using *csgE* and *csgF* primer pairs (Table B.1). Amplified products were visualized on %1 (w/v) agarose gel and gel images are shown Figure 2.9. pZs CsgG pLac/ara Kan vector was linearized with BamHI and visualized on %1 (w/v) agarose gel (Figure 2.10). PCR products were cloned with linearized vector using Gibson assembly method. pZs *csgGEF* pLac/ara Kan vector was shown in Figure C.5 and verified with sequence alignment (Figure D.4 and D.5).

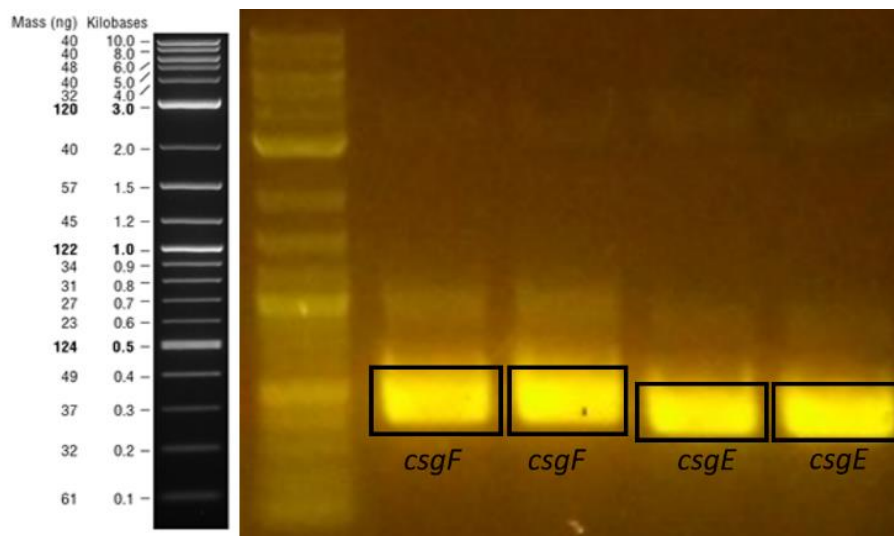


Figure 2.9: PCR amplified *csgE* and *csgF* gene fragments are observed at ~400bp as expected. *csgF* gene fragment is 479 bp and *csgE* gene fragment is 440 bp.

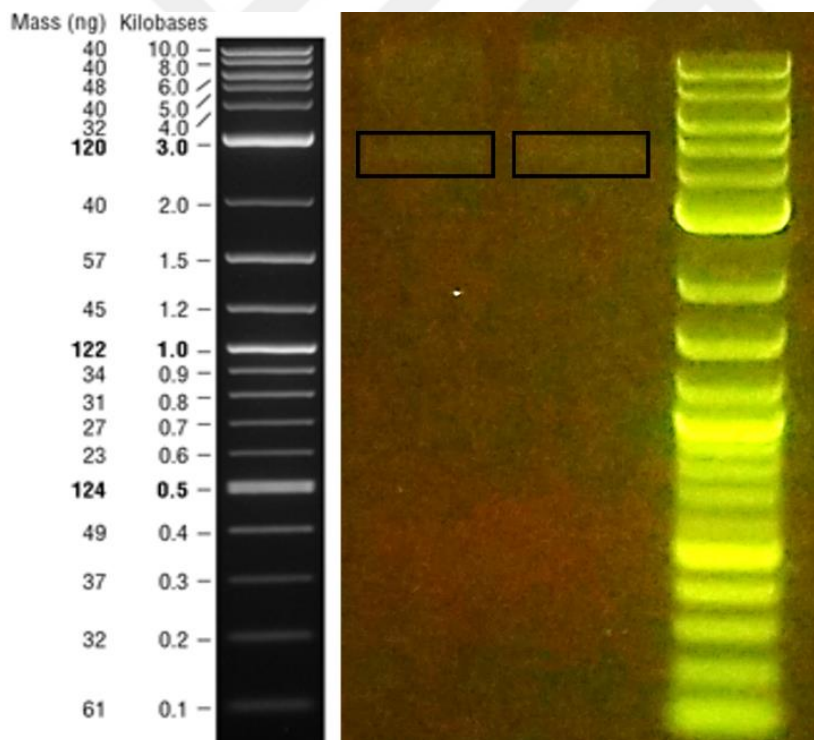


Figure 2.10: BamHI digested pZs CsgG pLac/ara Kan vector is observed between 4kb and 5kb ladder. Linearized vector was gel extracted from gel.

2.3.4. Crystal Violet Staining of Biofilms

pZa-tetO-ribo-csgBA-CmR and pZs csgGEF pLac/ara Kan vector was co-transformed into *DH5α PRO E.coli* strain. Biofilm formation of the transformed cell was determined by crystal violet assay. Overnight cultures were diluted 1:100, induced with the final concentration of 250ng/μl anhydrotetracycline (aTc), 1mM IPTG and %0.1 arabinose grown in 24 well plates containing LB medium for 3 days at 37 °C with 120 rpm shaking. This growth condition was chosen to prevent genomic expression of curli proteins. Induced engineered cells form thick and adherent biofilm in 24 well plates when compared to negative controls (*E. coli DH5α PRO* and Uninduced engineered cells) (Figure 2.11).

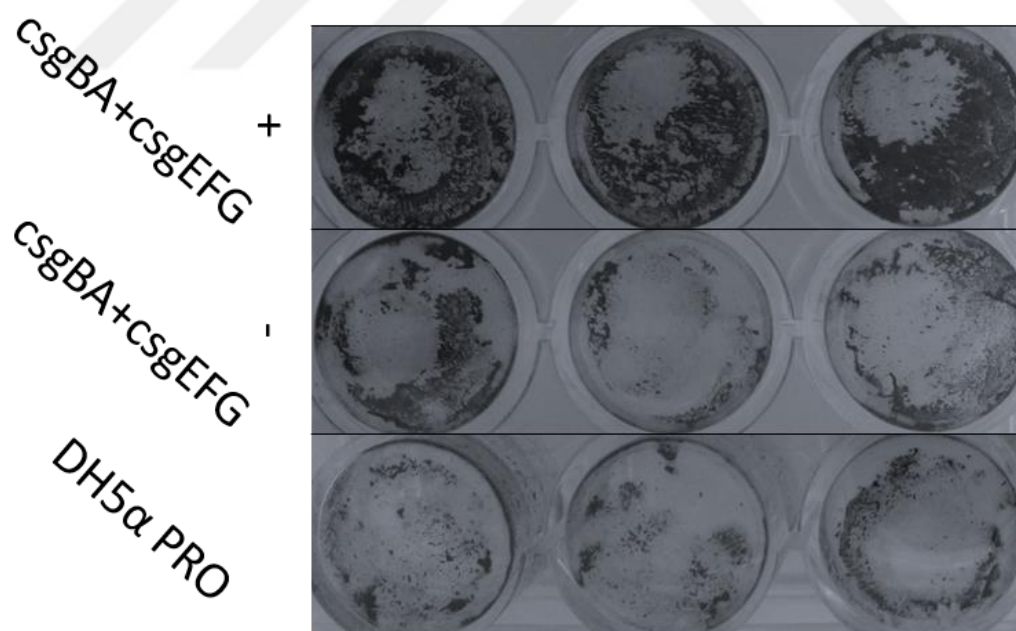


Figure 2.11: Crystal violet staining of the biofilms. First column contains csgBA and csgEFG containing induced cells, second column contains uninduced cells and third column contains *DH5α PRO* cells. Induced cells form thick and adherent biofilms.

2.3.5. Congo Red Staining of Curli Fibers

Congo red (CR) assay was performed to measure the amount of curli fibers quantitatively produced by pZa-tetO-ribo-csgBA-CmR and pZs csgGEF pLac/ara Kan containing *DH5α PRO E.coli*. Congo red gives absorbance at 480nm and binds to curli fibers. Consequently, loss of absorbance at 480 nm indicates the binding of Congo red to curli fibers.

Overnight cultures were diluted 1:100 and induced with appropriate inducers for 18 hours at 37 °C with shaking at 250 rpm. Then Congo red binding to curli fibers was measured by using the formula $-\text{OD}_{480}/\text{OD}_{600}$ (Figure 2.12).

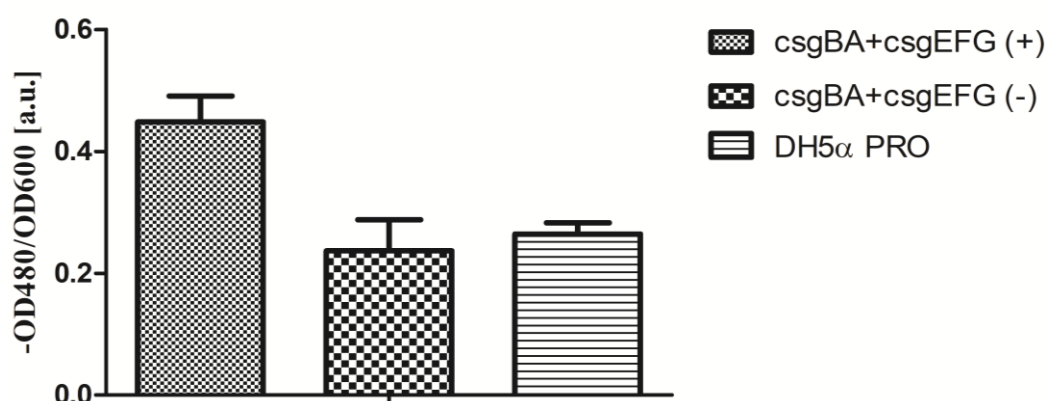


Figure 2.12: Congo red (CR) assay of curli fibers produced by engineered cells. First column contains csgBA and csgEFG containing induced cells, second column contains uninduced cells and third column contains *DH5α PRO* cells. Induced cells produce higher amount of curli fibers than uninduced cells and *DH5α PRO E.coli* cells.

2.3.6. Scanning Electron Microscopy (SEM)

Samples from congo red staining were used to prepare SEM samples. Curli fibers were observed around the engineered bacteria on SEM images (Figure 2.13).

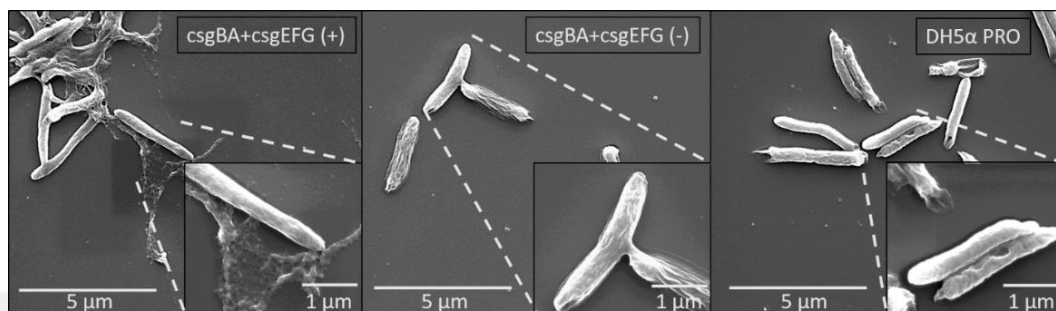


Figure 2.13: Scanning electron microscopy images of curli operon containing *DH5α PRO E.coli*.

2.4. Discussion and Conclusion

In this chapter, curli structural and accessory genes were successfully cloned into bacterial vectors with cut ligate method or Gibson assembly method. Only *csgA* and *csgB* containing vectors were used in mechanical characterization of curli fibers in chapter 4. They are under the control of aTc (anhydrotetracycline) inducible riboregulator system in which transactivating RNA (taRNA) and cisrepressor RNA (crRNA) controls the expression of a gene of interest as depicted in Figure 2.1. Transcription of taRNA and crRNA is controlled by TetO promoter which is induced by aTc molecule. In the absence of an inducer molecule, crRNA interacts with the ribosome binding site (RBS) to inhibit the translation of protein of interest. Meanwhile, the presence of inducer molecule triggers the production of taRNA sequence that disrupts the binding of crRNA to RBS site. In Figure 4.3, congo red staining shows the produced curli fibers in

csgA and csgB transformed cells. Δ csgBA *E.coli* cells were used as a control which doesn't produce any structural curli fibers and biofilms in Figure 4.3. csgA and csgB gene containing cells produce curli fibers, as observed on scanning electron microscopy images in Figure 4.2 and atomic force microscopy images in Figure 4.4.

Full operon csgBA and csgGEF were cloned into two independent vectors by Gibson assembly method in Figure C.3 and C.5 respectively. They are controlled by tetO promoter and Lac/Ara promoter respectively. In the presence of aTc molecule csgBA operon will be expressed and in the presence of IPTG/Arabinose csgGEF operon will be expressed. For the expression and secretion of curli fibers, whole operon should be expressed. Both plasmids and only csgGEF were transformed into *DH5 α PRO E.coli* cells. We used *DH5 α PRO E.coli* cell as a host cell because its genome contains inhibitor molecules (tetR and lacI) of tetO promoter and lacO promoter respectively and additionally host cell doesn't have kanamycin resistance gene which is already in pZs csgGEF pLac/ara Kan vector. In order to maintain the vector in the cell, host cell shouldn't contain same antibiotic resistance.

Our inducible vectors were characterized by crystal violet, congo red and SEM imaging. Crystal violet binds to extracellular complex materials and bacteria which enables us to quantify and qualify the biofilm formation on the surface of well plates. In Figure 2.11, we observed the thicker biofilm formation in the cells containing both csgBA and csgGEF with induction molecules. Uninduced and empty cells had less biofilm formation on the surface. Congo red binds to curli fibers which enables us to quantify the curli fiber formation of the cells. In Figure

2.12 we observed 2 times higher congo red staining in induced cells with respect to uninduced and empty cells. Observing congo red staining and crystal violet staining in control cells can be explained by the presence of csg operon genes in the genome. The growth conditions were used for the planktonic growth therefore; csg operon genes will not be opened. We also observed curli fibers in the induced cells containing csgBA and csgGEF operons in Figure 2.13.

In future studies, CRISPR-Cas9 system can be used to knock-out and even edit the full operon of *E.coli* [135] with designed fully controlled operon genes such as our designs. Nanofibers have variety of applications such as living adaptive coatings, environmental remediation, waste water processing, microbial fuel cell engineering, nanomaterial production in resource restricted environments, enzyme biocatalysis and microbiome engineering (Figure 1.6). Most recent developments in the engineering of biofilms for many various purposes were reviewed in the introduction part. In *E.coli*, curli proteins are the key elements of obtaining functional bio-nanomaterials on the surface and it is important to control the expression of operon genes.

2.6. Materials and Experimental Section

2.6.1. Materials

2.6.1. Preparation of Growth Medium and Buffers

To prepare LB (Luria-Bertani) media, 10g tryptone, 5g sodium chloride and 5g yeast extract were dissolved in deionized water and volume was completed to 1L. For agar media, 15g agar was added to 1L LB media.

To prepare 50XTAE (Tris-Acetic Acid-EDTA) media to use in agarose gel electrophoresis, 242 g Tris and 100ml of 0.5M EDTA solution were mixed and volume was completed to 1L.

To prepare the TSS (Transformation and Storage Solution) buffer for the preparation of competent cells, 5g Polyethyleneglycol (PEG), 1.5ml 1M $MgCl_2$ and 2,5 ml dimethyl sulfoxide (DMSO) were added to 50mL LB solution and filtered with 0,22 μ l filter. Solution was kept at -20 °C.

2.6.2. Methods

2.6.2.1 Plasmid Purification

Qiagen miniprep kit was used for plasmid isolation. Cells lysed with P1, P2 and N3 solutions were centrifuged 10min at 13000 rpm in microcentrifuge. Then DNA solution was loaded to QIAprep spin columns which were inserted into vacuum manifold. Spin columns were washed with 500 μ l PB buffer and 750 μ l PE buffer in order. Empty QIAprep spin columns were dried by centrifuge for 1 min. Plasmid DNA was eluted with 40 μ l water. Plasmid concentrations were measured with Nanodrop. Plasmid DNAs were stored at -20 °C.

2.6.2.2 Polymerase Chain Reaction (PCR)

Q5 High-Fidelity DNA Polymerase were used to amplify the genes using primers listed in Table B.1. All the reaction components (Table 2.1) were mixed on ice and transferred to BioRad Thermocycler and thermocycling conditions were applied according to primer annealing temperatures (Table 2.2).

Table 2.1: Reaction conditions

	Reaction components (μl)
10 μm forward primer	1,25
10 μm reverse primer	1,25
DNA (1 ng/μl)	1
Q5 polymerase	0,25
5x Q5 buffer	5
10 mM dNTP mix	0,5
Deionized water	16
Total volume	25

Table 2.2: Thermocycling Conditions

	Temperature	Time	Cycle
Initial denaturation	98	30 sec	
Denaturation	98	10 sec	30
Annealing	59-72	20 sec	
Extension	72	20 sec	
Final extension	72	7 min	

2.6.2.3 Agarose gel electrophoresis

%1 (w/v) agarose gel was prepared in 1XTAE buffer. 0.5g of agarose was mixed in 50ml 1XTAE and then boiled in microwave for 2-3min. SYBR Safe was added into agarose gel after cooling hot agarose solution for imaging of the DNAs. 1XTAE was also used for running buffer on horizontal electrophoresis apparatus. DNA samples were loaded on agarose gel with purple loading dye (NEB) and agarose gel was run at 120V for 30 minutes. 2-log DNA ladder (NEB) or 50bp DNA ladder (NEB) were used to determine the size of DNA samples.

2.6.2.4 Gel Extraction

Commercial gel extraction kit (MN) was used to extract DNA from agarose gel. DNA samples were sliced from agarose gel and put into an Eppendorf tube. Agarose gel was melted by addition of gel solubilization buffer (NT1) at 50 °C for 10 min. Then DNA solution was loaded to MN spin columns which were inserted into vacuum manifold. Spin columns were washed with 700 µl NT3 buffer. Empty MN spin columns were dried by centrifuge for 1 min. Extracted DNA was eluted with 20 µl water. Plasmid concentrations were measured with Nanodrop. Plasmid DNAs were stored at -20 °C.

2.6.2.5 Endonuclease Digestion Reactions

DNA samples which will be ligated were digested with same endonucleases (NEB). Reaction was set up as in Table 2.3 and incubated at 37 °C for 1 hour. Digested products were visualized on agarose gel and extracted from it.

Table 2.3: Restriction-digestion reaction set-up

Component	Amount
DNA	200 ng
Enzyme 1	0,2 µl
Enzyme 2	0,2 µl
10x cut smart buffer	2 µl
Deionized water	Up to 20 µl

2.6.2.6 Ligation Method

Gel extracted DNA samples which were digested with endonucleases were mixed using molar ratio of 1:3 (vector: insert) to ligate parts. Ligation of the sticky ends were set up as in table 2.4 and incubated at room temperature for 5-10min.

Table 2.4: Restriction-digestion reaction set-up

Component	Amount
T4 DNA Ligase Buffer (10X)*	2 μ l
Vector DNA (4 kb)	50ng
Insert DNA (1 kb)	37.5ng
T4 DNA Ligase	1 μ l
Deionized water	Up to 20 μ l

2.6.2.7 Gibson Assembly Method

In this assembly method, primers were designed with 30 bp matching with the adjacent DNA fragment. Then DNA fragments were amplified with primers. To avoid undesired products, fragments were purified from gel. Then DNA fragments and components (T5 endonuclease, Phusion DNA polymerase and Taq DNA ligase) of the Gibson assembly were combined and incubated at 50 °C for 1 hours. T5 endonuclease creates single stranded 3' DNA by degrading 5' end of the DNA. Phusion DNA polymerase fills the gaps between overlapping DNAs. Taq DNA ligase connects the annealed DNA fragments covalently for continuous DNA. After reaction, DNA fragments were transformed into chemically prepared competent cells.

2.6.2.8 Competent Cell Preparation

E.coli cells were inoculated from glycerol stock or LB agar plate for overnight growth at 37 °C. Next day, culture was diluted into 1/100 and grown until OD₆₀₀ reaches between 0.4-0.6 at 37 °C with shaking at 250 rpm. Cells were centrifuged down for 10 minutes at 10000 rpm at 4 °C. Cell pellets were concentrated into 10 fold with TSS buffer and were divided into 100 µl aliquots to store at -80 °C.

2.6.2.9 Transformation of Plasmids to E. Coli Component Cells

Cells were taken from -80 °C and thawed on ice 30 min. LB agar plates with appropriate antibiotic were taken from +4 °C for warming up. Ligation mixture (10pg-100ng) were added to cells (20-50µl) and gently mixed by flicking the tube. Reaction mixture were incubated on ice for 20-30 mins. Then cells were exposed to heat shock at 42 °C, heat blocked for 45 seconds and immediately put back on ice for 2 mins. 400 µl LB was added to tube and incubated for 1hours at 37 °C. Finally, 100 µl was spread on LB agar plate and plate was incubated at 37 °C overnight.

2.6.2.10 Scanning Electron Microscopy Imaging

Curli fiber production was induced with appropriate inducer (aTc, arabinose or IPTG) under static or dynamic culture condition. For sample preparation, biofilm was washed with ddH₂O and PBS and fixed with 2.5 % glutaraldehyde overnight at +4 °C. Next day, samples were dehydrated by washing twice in each concentration of ethanol in a graded series (%30, %50, %70, %99) for 5 min followed by sputtering 5nm Au/Pd alloy.

CHAPTER 3

Genetically Engineered Conductive Curli Nanofibers

This work is partially described in the following publication[136]:

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3.1. Objective

Bacterial biofilms are promising tools for functional applications as bionanomaterials. They are synthesized by well-defined machinery, readily form

fiber networks covering large areas, and can be engineered for different functionalities. In this work, bacterial biofilms have been engineered for use as conductive biopolymers to interface with electrodes and connect bacterial populations to electronic gadgets. Bacterial biofilms are designed with different conductive peptide motifs, as the aromatic amino acid content of fused peptide motifs has been suggested to contribute to electronic conductivity by influencing monomer stacking behavior. To select best candidates for constructing conductive peptide motifs, conductivity properties of aromatic amino acids are measured using two different fiber scaffolds, an amyloid-like fiber (ALF) forming peptide, and the amyloidogenic R5T peptide of CsgA protein. Three repeats of aromatic amino acids are added to fiber-forming peptide sequences to produce delocalized π clouds similar to those observed in conductive polymers. Based on the measurements, tyrosine and tryptophan residues provide the highest conductivity. Therefore, the non-conductive *E. coli* biofilm is switched into a conductive form by genetically inserted conductive peptide motifs containing different combinations of tyrosine and tryptophan. Finally, synthetic biofilm biogenesis is achieved with conductive peptide motifs using controlled biofilm production. Conductive biofilms on living cells are formed for bioelectronics and biosensing applications

3.2. Introduction

Long range electron transfer has been a major topic of interest in peptide and protein research for decades [137-140]. Investigations of electron transfer in biologically important molecules such as photolysis [141], cryptochromes [142], phototropins [143] and ribonucleotide reductase [144, 145] have demonstrated

that aromatic amino acids such as tyrosine and tryptophan are vital for catalytic activity in many enzymes [146-149]. Amino acid composition and structure of the peptides contributes to electronic conductivity [150], and many studies have focused on electron transfer between aromatic amino acids using peptide motifs. Conjugated molecules with low electron tunneling barriers typically exhibit superior conductivity, and peptide and protein based conductive materials are promising for several applications [151-154]. In nature, electron transfer is necessary for the circulation of energy in the biosphere. *Geobacter sulfurreducens* and *Shewanella oneidensis* have conductive nanowire structures to transfer electrons to oxidized metals as final electron acceptors during anaerobic respiration [155, 156]. Conductivity in biology utilizes many of the design principles that are found in conductive polymers [157]. For example, polyacetylene is a conjugated polymer with free mobile π electrons, the doping of which results in strong conductivity. Similarly, the C-terminal of the PilA protein takes part in the biofilm formation of *G. sulfurreducens* and has aromatic clusters playing important role in conductivity [158]. It has been shown that, when aromatic amino acids are replaced by alanine (which lack functional side groups) in the PilA nanowire, *G. sulfurreducens* becomes unable to transfer electrons and to reduce oxidized metals [159]; moreover, recent studies on genetic engineering of the PilA protein increased the conductivity of PilA nanofibers through the substitution of amino acids with tryptophan residues [160]. This demonstrated the importance of the aromatic amino acids in conductivity and highlighted their potential to be incorporated in biofilm-forming protein structures to transfer electrons to electrodes [161-163]. Due to the difficulties on the growth of *G.*

sulfurreducens, which needs an electron acceptor under anaerobic conditions for growth [164, 165] and can only survive at oxygen concentrations below 10% in the environment [166], the development of novel interface systems utilizing well-documented model organisms would contribute greatly to the potential of bioconductive materials in device applications. In particular, interfacing cells with electrodes can provide many opportunities in microbial fuel cell applications, biosensors, electrogenetic applications, light-harvesting applications, and synthetic biology applications [167].

Escherichia coli is commonly used as the go-to organism in synthetic biology applications, as it expresses a natural fiber system (the *curli* group of proteins) that is capable of secreting and displaying specific protein sequences [168, 169]. In recent reports, *E. coli* was engineered to carry electrons from its electron pool to the cell membrane by the use of a pathway electron conduit borrowed from *S. oneidensis* [170, 171]. This non-native pathway was domesticated and coupled with a native *E. coli* cytochrome, NapC [170]. Studies revealed the possibility of pumping out electrons from *E. coli* cytosol to the external environment [170]. However, to enable such direct electron transfer with electrode surfaces, there is a need to interface cells with synthetic electrical functionalities with electrode surfaces. In addition to cell-cell communications in bacteria (*i.e.* quorum sensing) [172], recent studies have shown that bacteria in biofilms can communicate using electrical signals. The presence of ion channel-mediated electrical communication was subsequently demonstrated in bacteria, and changes in bacterial membrane potential were found to alter their metabolism during these communication events [173]. Given these recent developments, the design of a conductive interface

would also be beneficial to control charge transfer into bacterial biofilm communities, potentially controlling the behavior of the entire bacterial population. In addition, the secretion of such an interfacing material from living cells will greatly simplify the design of biofilm-interfacing devices by eliminating additional surface modification steps that are necessary to assemble the bacteria on electrode surfaces. Using bacterial biofilm instead of purified fiber will save time from isolation and even in vitro assembly of fibers. Biofilm cells produce already self-assembled curli fibers on the surface. It is easy to work on biofilm interface and no additional step is required for assembly of fibers whereas purification and assembly of fibers in vitro is required many steps and chemicals.

Nanofibers exhibiting a broad range of biological functions have been synthesized using new nanoscale assembly methods [174]. One particular technique entails the production of nanofibers through high speed gyration and the subsequent modification of the nanofiber surface by genetically functionalized fusion proteins, which allows the mass production of complex nanofiber structures. The secretion of nanofibers or their precursors directly from genetically engineered bacteria is another method for the mass production of functional fibers. Biofilm protein fibers are very promising interfacing materials between living cells and abiotic entities such as electrodes and surfaces [175]. Many bacteria can form biofilms in response to different stimuli, including starvation, toxic substances, extracellular materials, social competition or other stress conditions [176-179].

E. coli forms biofilms using a curli operon that is formed by *csgBA* and *csgDEFG* operons. *csgBA* codes for CsgA and CsgB proteins, which form the backbone of the biofilm structures, whereas *csgDEFG* codes the accessory proteins for biofilm

production [180]. *E. coli* has two structural curli proteins: the major subunit CsgA and minor subunit CsgB [181]. Among these proteins, CsgB, serves as a nucleation site for CsgA protein to attach and form the nanofiber network [182, 183]. We aimed to engineer the CsgA protein for functional peptide fusion. Intermolecular interactions between curli subunits allow them to form large hierarchical networks, which makes them promising candidates as bionanomaterials [184]. Repeating units of CsgA proteins form intermediate-length curli fibers [185] and can be modified with various peptide tags for the surface expression of inorganic materials [117]. Previously, CsgA was fused with various peptide motifs and the functionality of these peptides was demonstrated [186]. Curli system also enables the cell populations to strongly adhere on surfaces, enabling a robust contact interface [185, 187].

3.3. Results

3.3.1 AFM Characterization of Peptides

The effect of addition of different amino acids to the fiber forming peptides were investigated by using AFM (Atomic Force Microscopy). All peptides were drop-casted on mica surfaces and dried overnight. Dried samples were imaged by AFM tapping mode using silica probe. All the peptides form fibers with different morphologies (Figure 3.1).

3.2.2 SEM Characterization of Peptides

Peptide samples were prepared on silica wafers for SEM imaging. Different morphologies were observed when the scaffold peptides were substituted with the addition of aromatic residues. There was not a general trend for morphology

changes as a function of the type of the amino acids in different scaffold peptides (Figure 3.2).

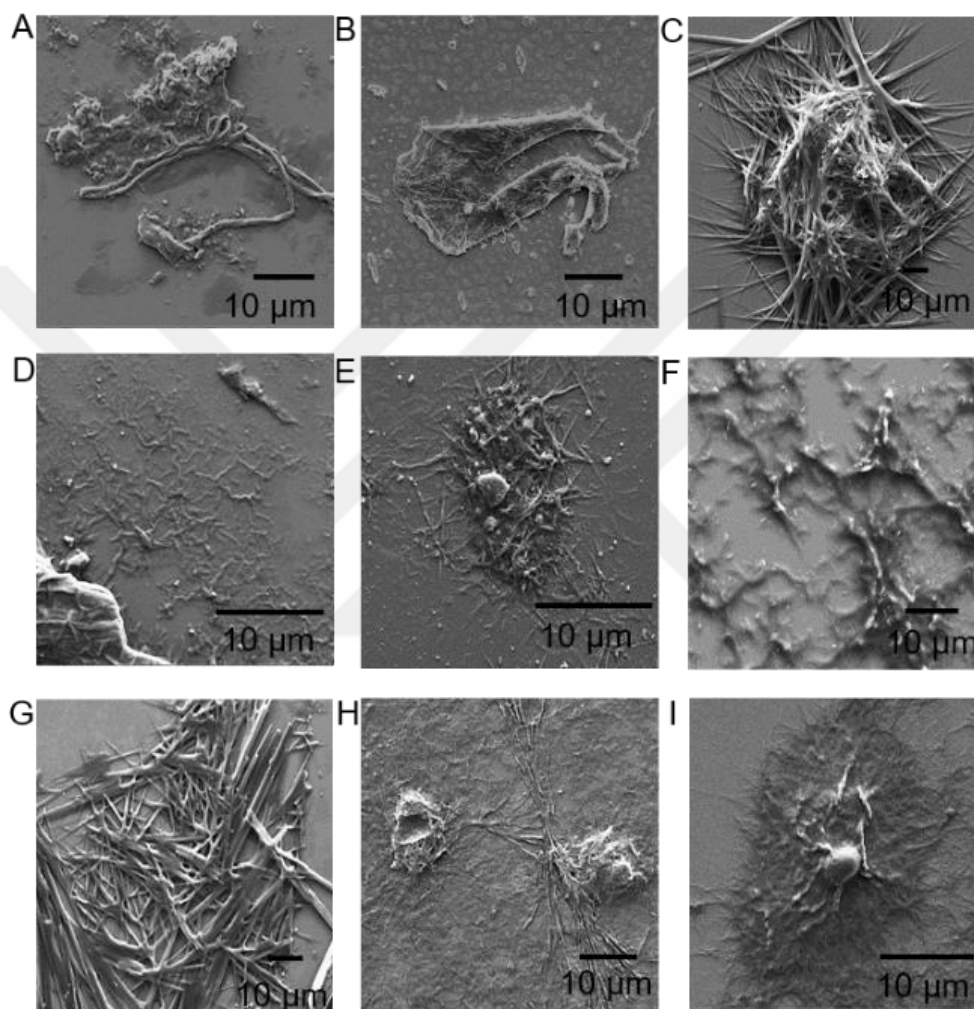


Figure 3.1: Scanning electron microscopy (SEM) images of the designed peptides on SiO₂. Amyloid-like fiber (ALF) (A), ALF3W (B), ALF3Y (C), ALF3H (D), ALF3F (E), R5T (F), R5T3W (G), R5T3Y (H), and R5T3H (I).

3.2.3 Conductivity Curves of Peptides

The measurements were taken on three different electrodes for each sample. Each curve represents the average of three independent samples. Samples were left for

drying in high humidity chamber. Figure 3.4 and 3.5 show a close-up view of the conductivity of the peptides shadowed by high conductivity values. R5T3W, R5T3Y, and ALF3W samples showed high-current voltages with non-linear behavior between -5V to and +5V (Figure 3.3). The rest of the samples showed low-current voltages with linear behavior between -5V and +5V (Figure 3.4 and 3.5).

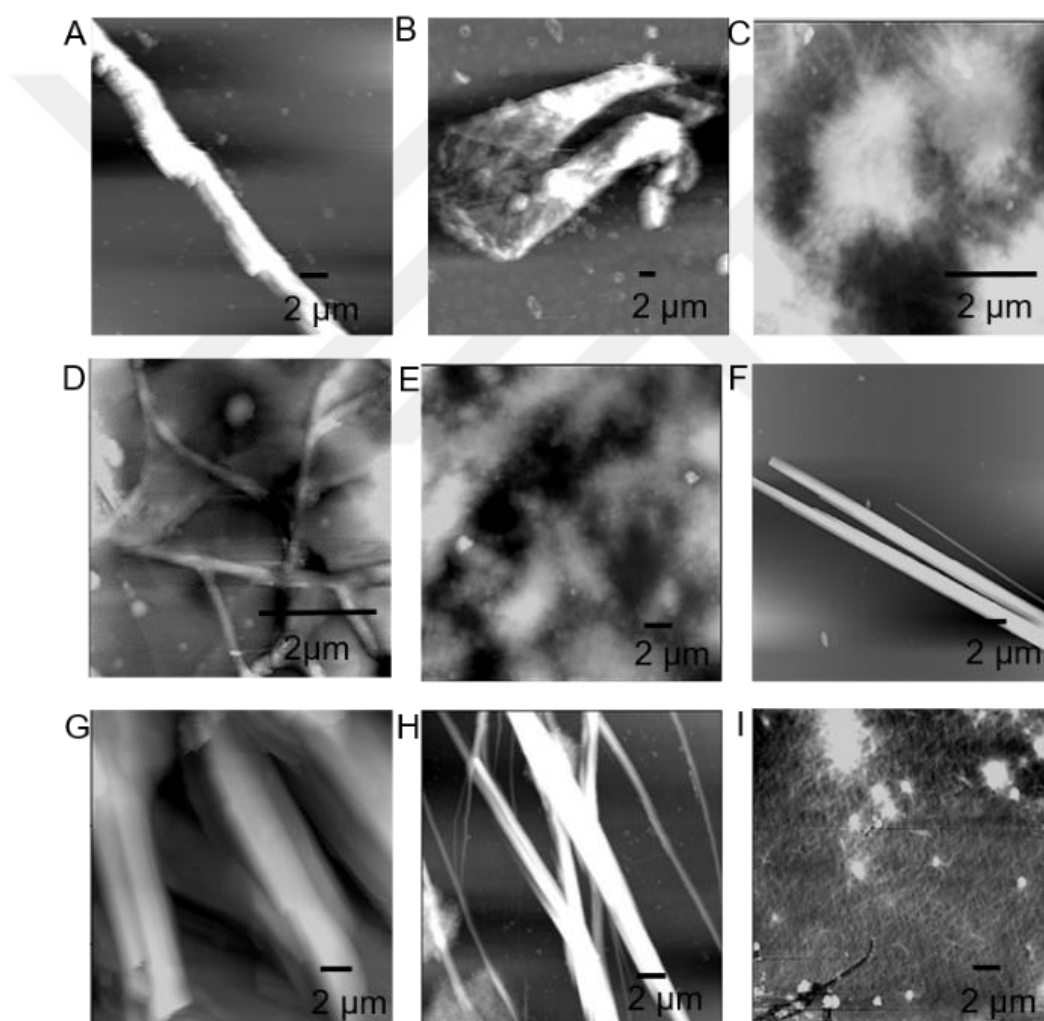


Figure 3.2: Atomic force microscopy (AFM) images of the designed peptides on mica. Amyloid like fiber (ALF) (A), ALF3W (B), ALF3Y (C), ALF3H (D), ALF3F (E), R5T (F), R5T3W (G), R5T3Y (H), and R5T3H (I).

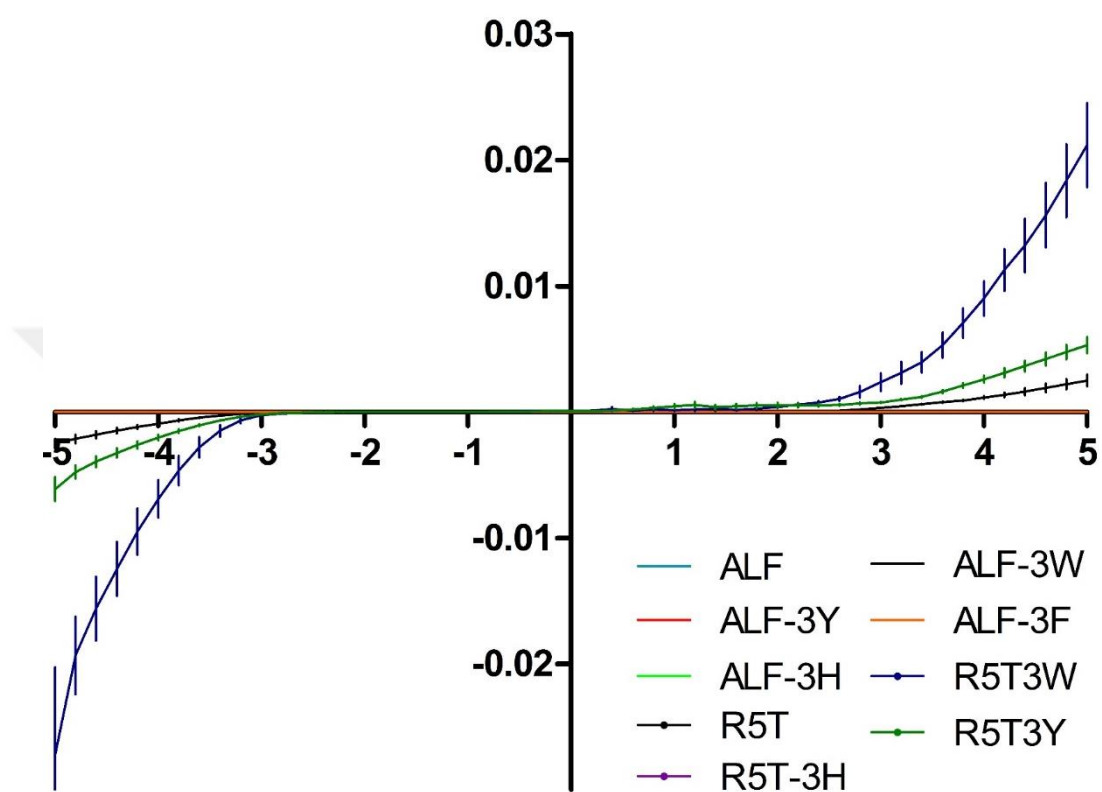


Figure 3.3: Conductivity curves for the peptide nanofibers.

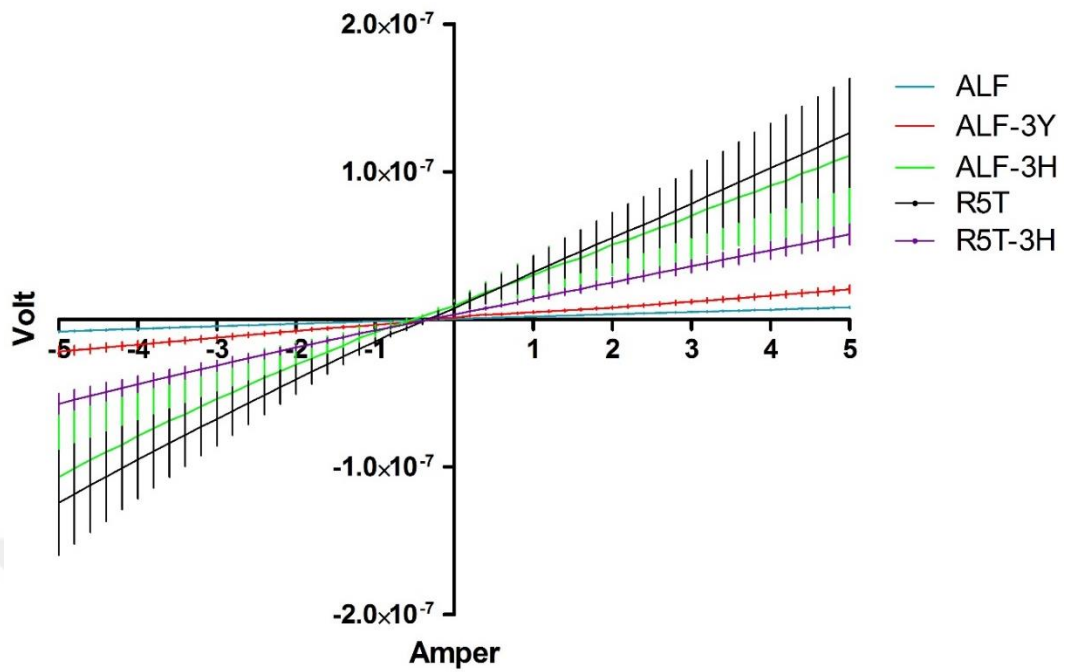


Figure 3.4: Conductivity curves for ALF, ALF-3Y, ALF-3H, R5T and R5T3H.

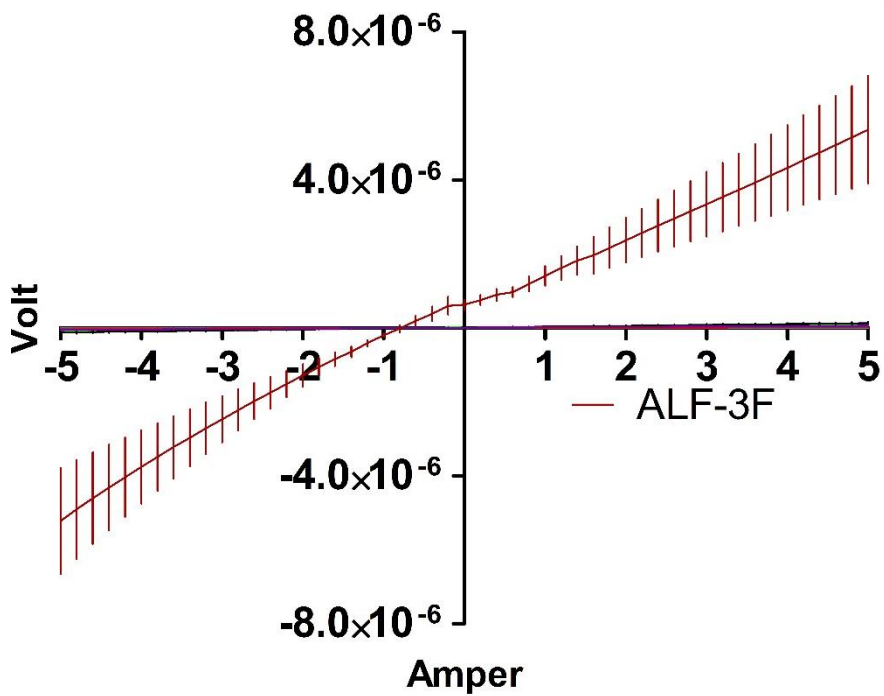


Figure 3.5: Conductivity curves for ALF-3F.

3.2.4 Crystal Violet Staining

Crystal violet assay was performed to confirm biofilm formation by all engineered curli producing cells in the presence of inducer aTc (anhydrotetracycline). Assay performed in triplicate. All the engineered cells forms thick and adherent biofilms in 24 well plates when compared to negative controls (*E. coli* MG1655 *PRO ompR234 ΔcsgBA* and empty pZA vector-transformed *ΔcsgBA* cells) (Figure 3.6).

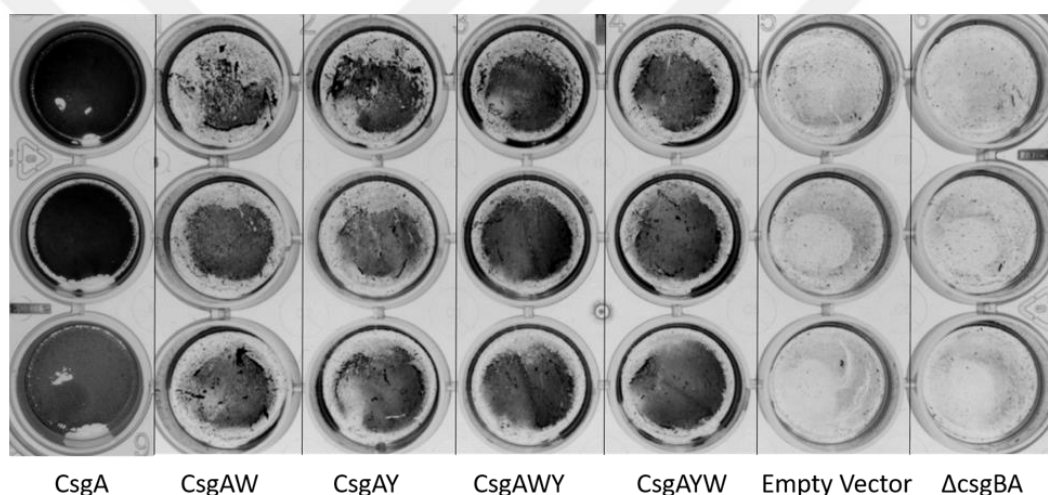


Figure 3.6: Crystal violet (CV) staining of the biofilms were formed by *E.coli* *ΔcsgBA* cells containing modified CsgA (CsgAW, CsgAWY, CsgAY, and CsgAYW) and CsgA in the presence of aTc. Crystal violet staining allows the quick observation of biofilm formation. No biofilms were formed by non-transformed and empty vector-transformed *E.coli* *ΔcsgBA* cells. Cell cultures were grown in M63 medium with glucose in 24-well plates at 30 °C with no shaking for 3 days.

3.2.5 Congo Red Staining

The amount of curli fibrils produced by recombinant bacteria were also characterized quantitatively and qualitatively by Congo Red (CR) assay. Red color was observed in induced cells when they are incubated with CR (Figure 3.7). Further, absorbance of CR was performed at 480 nm. Congo red binding to modified CsgA proteins were ~1.33-fold more than unmodified CsgA protein (Figure 3.7).

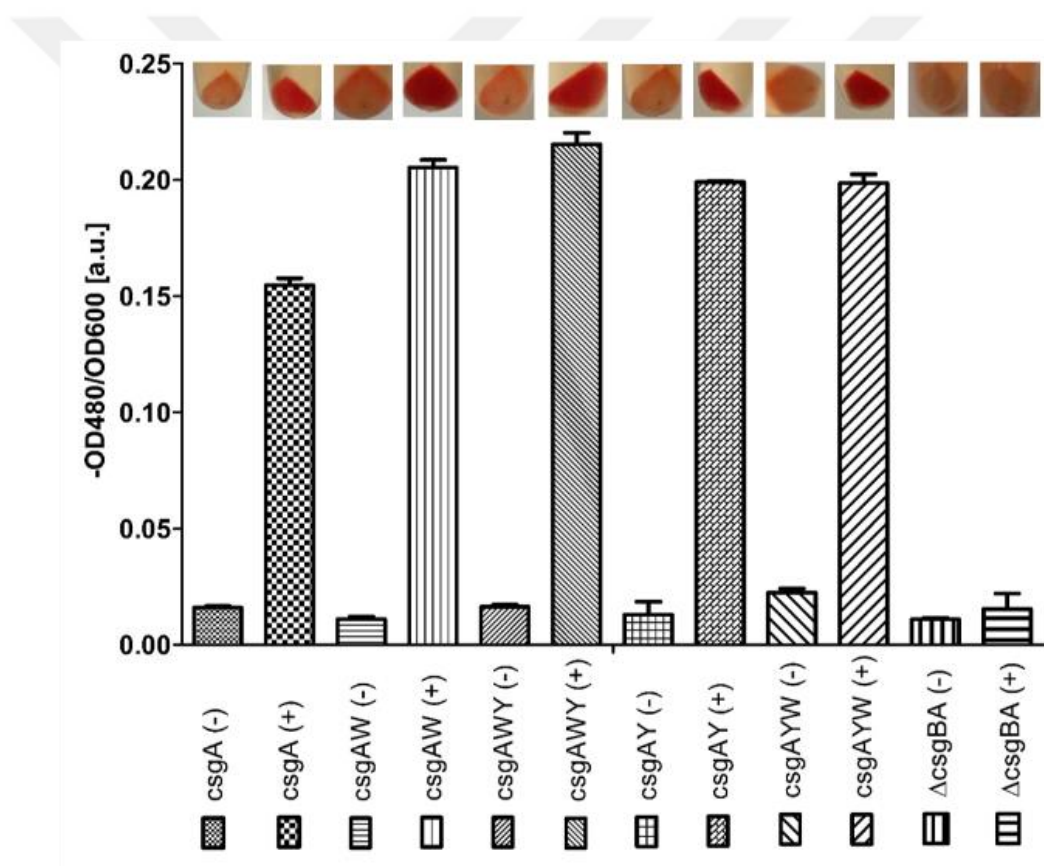


Figure 3.7: Congo red (CR) assay of biofilms showed that engineered curli proteins are being produced in the cells.

3.2.5 SEM Images of Conductive Fibers

Assembly and morphology of conductive curli fibers were observed around the engineered bacteria on SEM images (Figure 3.8). SEM data showed that

conductive fibers were 2-2.5 μm in length and 25-35 nm in thickness. This length shows that 5 to 7 individual *curli* fibers might come together to form fiber bundles.

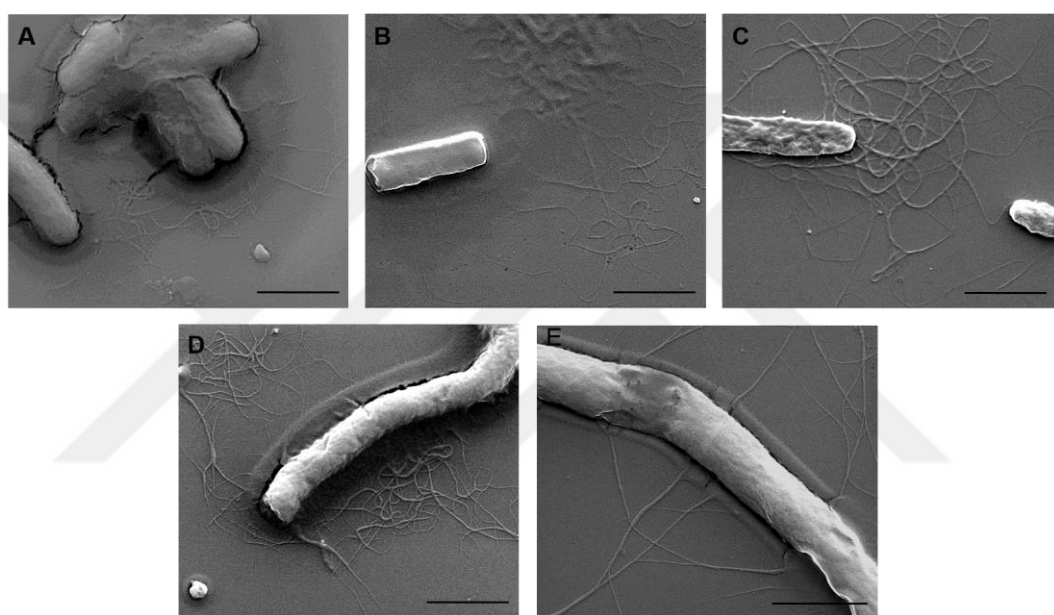


Figure 3.8: Scanning electron microscopy images of the CsgA fibers and the designed conductive CsgA fibers produced by *E.coli* ΔcsgBA cells: CsgA (A), CsgAW (B), CsgAY (C), CsgAWY (D), and CsgAYW (E). Scale bars are 2 μm .

3.2.6 TEM Images of Conductive Fibers

Further, Conductive fibers thickness were characterized with TEM images of fibers (Figure 3.9). The much thicker bundles $\sim 15\text{-}20$ nm in TEM images were observed than single CsgA monomers $\sim 3\text{-}4$ nm in the literature [188].

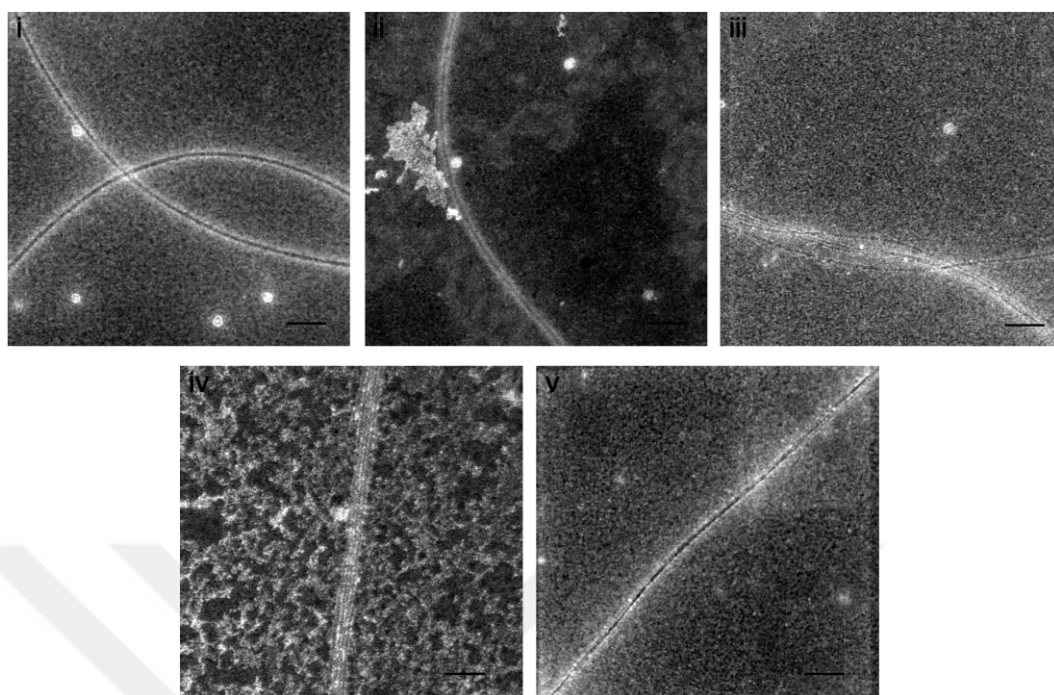


Figure 3.9: Transmission electron microscopy images of the CsgA fibers (i) and the designed conductive CsgA fibers produced by $\Delta csgBA$ E.coli cells: CsgA-W (ii), CsgA-Y (iii), CsgA-WY (iv), and CsgA-YW (v). Diameters of the fibers were about 12-15nm and many subunits came together to form thick fibers. Scale bars are 100 nm.

3.2.7 Conductivity Measurement of Biofilms

Linear ohmic behavior in all biofilms was observed in conductivity measurement by probe station analysis. (Figure 3.10). CsgAW, CsgAY, CsgAWY and CsgAYW motifs exhibited conductivity values about 7.61, 8.13, 5.86, and 3.27 fold higher than *E.coli* $\Delta csgBA$ cells and 4.08, 4.37, 3.14, and 1.76 fold higher than unmodified CsgA biofilms, respectively (Figure 3.10).

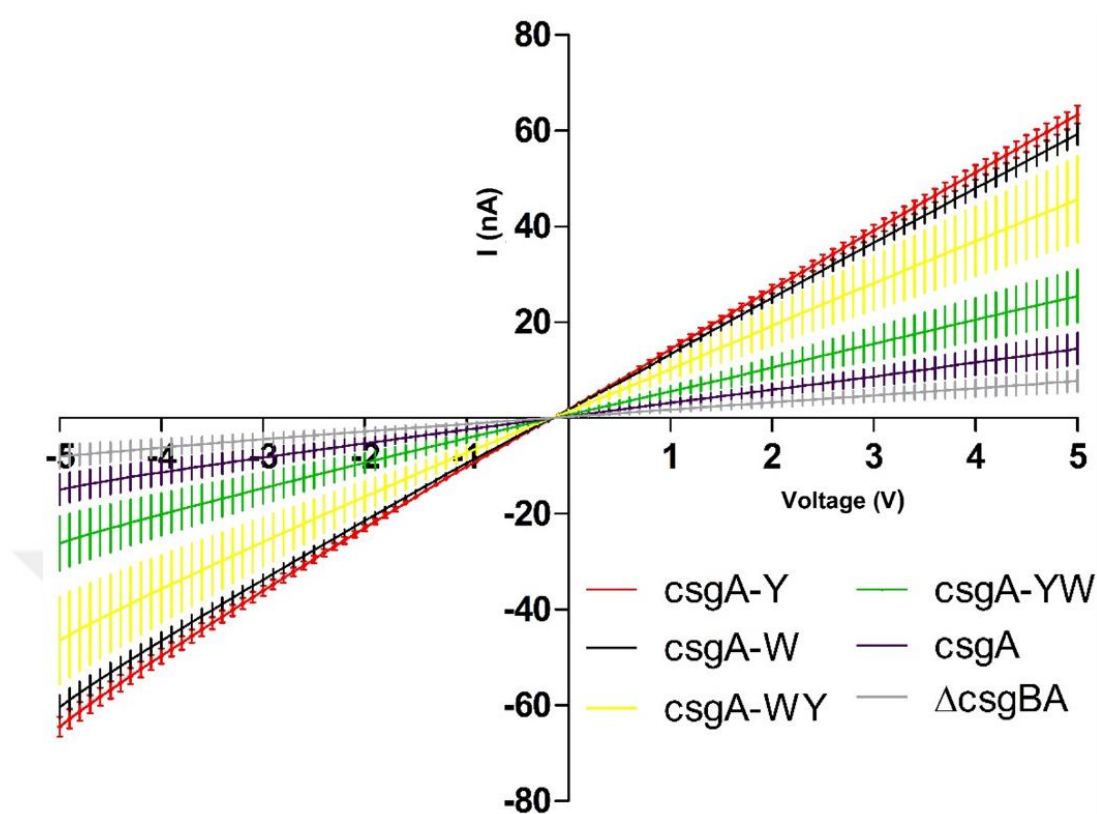


Figure 3.10: Conductivity curves for CsgA and modified CsgA proteins representing functional engineered amyloids fibers. Measurements were taken on three different electrodes for each sample. Curves represent the average of three independent samples.

Inter digitated gold electrodes were used to measure conductivity in biofilms (Figure 3.12). After each preparation of electrodes were checked by probe station (Figure 3.13). Figure 3.11 shows the biofilm added electrode after measurement of conductivity.

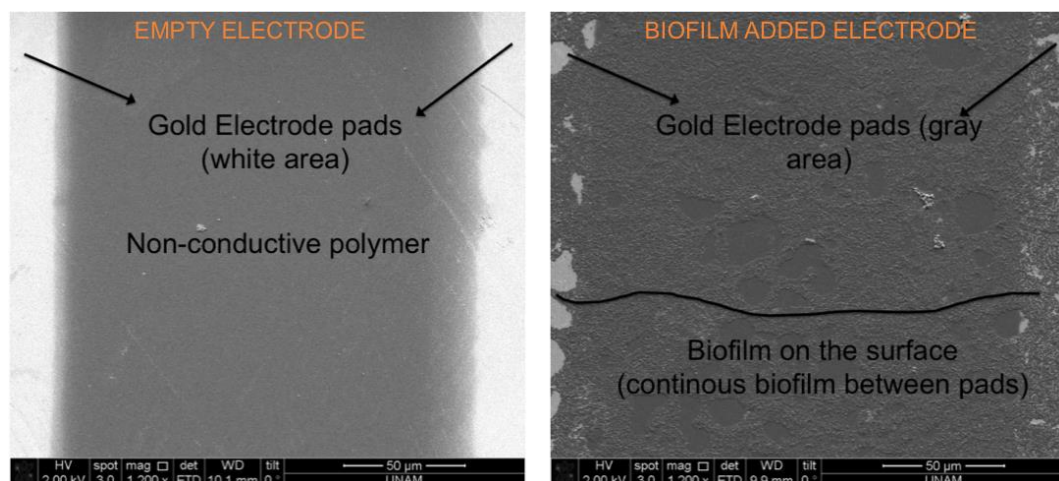


Figure 3.11 Interdigitated gold electrodes before and after the addition of the biofilms on surface for conductivity measurements.

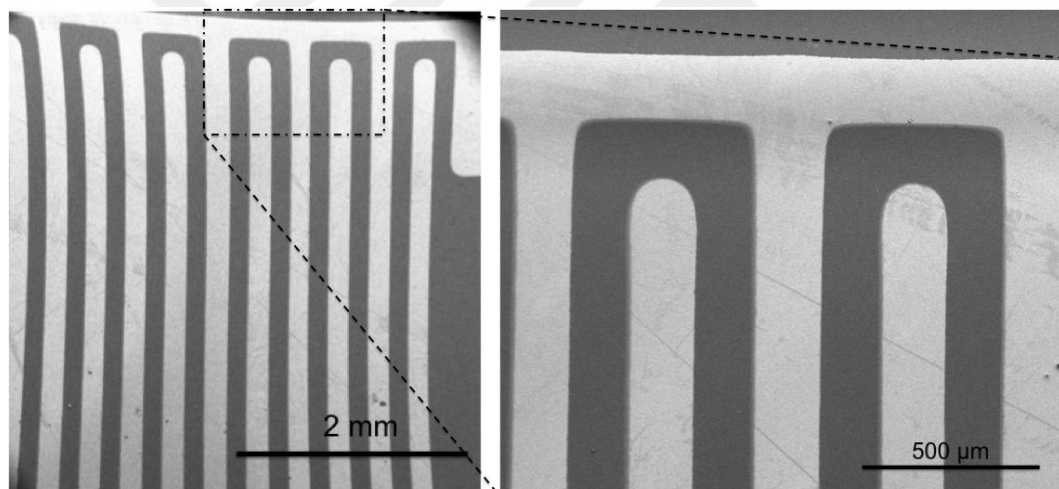


Figure 3.12 Empty interdigitated electrode and its detail showing the successful deposition of the gold through the mask used.

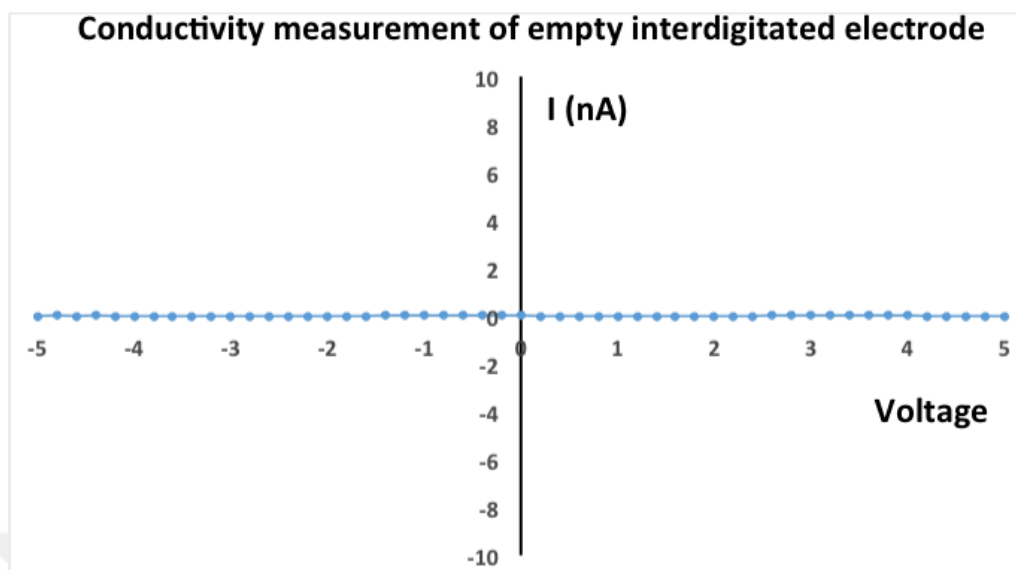


Figure 3.13: Conductivity measurement of the empty electrode.

3.2.8 3D Structure Modelling of Modified CsgA Protein Monomers

To gain insights into the conductivity of engineered CsgA proteins containing conductive peptide motifs, template-based tertiary structure modelling was performed using RaptorX, a web-based server, to obtain their 3D structure, because homology modelling is not available to model CsgA protein (Figure 3.14).

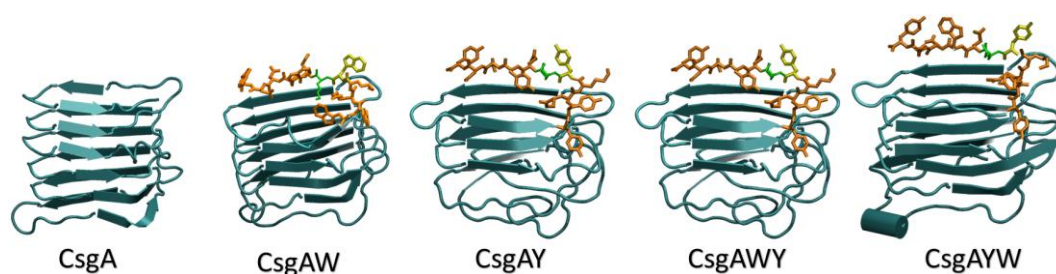


Figure 3.14: Molecular models of CsgA protein and its conductive motif-fused variants.

3.4. Discussion

In this study, given the previous findings on the role of aromatic amino acids in conductivity [137, 159, 160, 189-191], we first tested the electrical conductivity of four aromatic amino acids; tyrosine, tryptophan, phenylalanine and histidine, on two different fiber-forming scaffold peptides; an amyloid-like fiber (ValGlyGlyLeuGly) [192] peptide and the amyloidogenic R5T peptide (SerValAspValThrGlnValGlyPheGly) of CsgA protein [193], to form seven distinct, potentially conductive peptide sequences (Figure 3.15 and Table 3.1). Synthetic peptide scaffolds helped us to screen the conductivity of aromatic amino-acids for best candidates to be used in protein engineering.

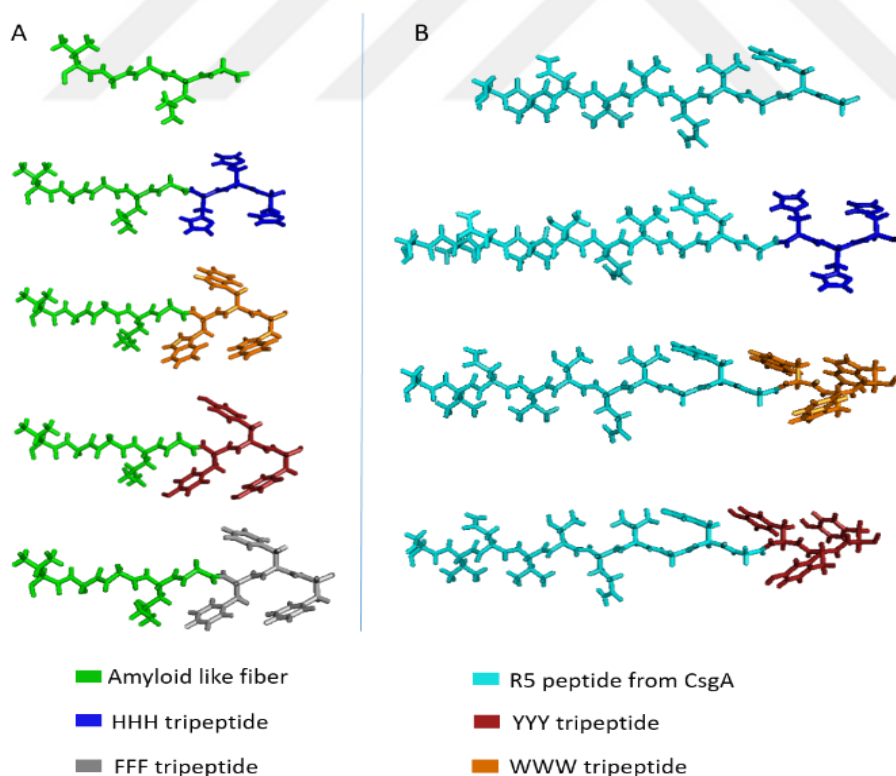


Figure 3.15 Molecular models of designed synthetic peptides. A. Amyloid-like fiber templated peptides. B. R5T peptide templated peptides.

These aromatic amino acids had similar structures to conductive polymers; as such, all peptide constructs were expected to be conductive to some degree. To probe the effect of the addition of different aromatic amino acids on the assembly of the peptide fibers, the morphologies of the peptides were investigated with atomic force microscopy (AFM) (Figure 3.1) and scanning electron microscopy (SEM), as demonstrated in Figure 3.2. Samples for SEM imaging were prepared on silica wafers. The original scaffolds on Figure 3.2A and Figure 3.2F were observed to form fiber structures, as is expected from previous findings [192, 193]. The addition of aromatic residues resulted in different morphologies. We did not observe a general trend for morphology changes as a function of the type of the amino acids in different scaffold peptides. It is assumed that fiber-making scaffold peptide sequences, in conjugation with the added amino acids, are controlling the final aggregate/fiber morphology. In all of the cases, the addition of aromatic amino acids to the scaffolding peptides did not preventing the formation of large peptide fibers or aggregates.

Table 3.1: A series of peptides where synthesized and tested in our conductive measurements. ^a Amyloid like fiber described by del Marcato et al. [192] ^b R5 peptide from CsgA protein described by Lembre et al. [193] ^cGRAVY: Grand average of hydropathicity.

<i>Groups</i>	<i>Peptide</i>	<i>Sequence</i>	<i>Gravy index^c</i>
1	ALF ^a	VGGLG	1.360
1	ALF3H	VGGLGHHH	-0.350
3	ALF3W	VGGLGWWW	0.512
2	ALF3Y	VGGLGYYY	0.362
2	ALF3F	VGGLGFFF	1.900
1	R5T ^b	SVNVTQVGFG	0.610
1	R5T3H	SVNVTQVGFGHHH	-0.262
3	R5T3W	SVNVTQVGFGWWW	0.262
2	R5T3Y	SVNVTQVGFGYYY	0.169

Conductivity of peptide fibrils were investigated using probe station measurements under the configuration shown in Figure 3.16. In order to explore the conductive properties of peptide fibrils, their solutions were deposited on gold electrodes. Electrical conductivities of the peptide samples are provided in Figure 3.3-5. High-current voltages were obtained for R5T3W, R5T3Y, and ALF3W samples, which also exhibited non-linear I-V characteristics. The rest of the samples showed a linear ohmic behavior in the range of -5V to 5V, suggesting that their Schottky barrier I/V may be higher or that they are more insulating in this region (Figure 3.4-5). It is clear that tryptophan and tyrosine insertions have superior conductivities in comparison to other insertions (Figure 3.3) [147, 189, 194].

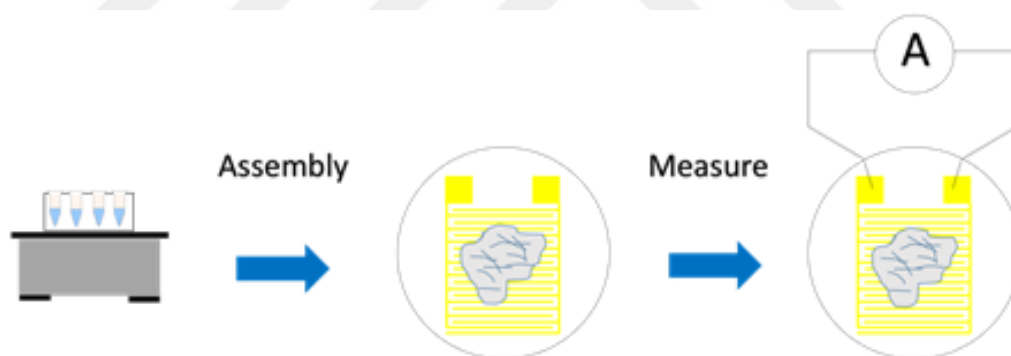


Figure 3.16 Assembly of fibers and conductivity measurement of fibers using a probe station apparatus.

According to these findings, tyrosine and tryptophan were selected for the functionalization of the curli nanofibers to convert them into conductive fibers. The C-terminal of the PilA protein (Figure 3.17A) features a conductive motif composed of 5 aromatic amino acids and 6 charged amino acids, both of which are crucial for fiber conductivity [160, 195]. In a recent study, the insertion of

tryptophan residues in the C-terminal of *G. sulfurreducens* PilA protein resulted in increased nanofiber conductivity [160]. One of the critical points in the engineering of conductive protein fibers is to increase conductivity through the insertion of additional amino acid residues. Consequently, we mimicked the conductive peptide motif of PilA as presented on Figure 3.17A, and modified this initial sequence with four different peptide designs shown in Figure 3.17B. These peptides were genetically fused with the 3' end of the *csgA* gene. Charged amino acids were kept at the same positions in the modified CsgA design for conductive nanofiber formation, while the aromatic amino acids were replaced by tyrosine or tryptophan (Figure 3.17B).

Gene fragments used in the biosynthesis of conductive nanofibers were amplified from the *E. coli* genome. DNA sequences of the designed conductive motifs were added to the 3' end of gene fragments by PCR in conjunction with overlap extension. Recombinant genes were cloned into pZa-tetO-CmR using the cut ligate method, vector maps can be seen in the Appendix C. The inducible expression of recombinant proteins was therefore achieved through the pZa-tetO-CmR vector. Expressions of CsgAW, CsgAY, CsgAWY and CsgAYW proteins are under tight regulation by an anhydrotetracycline (aTc) inducer-responsive riboregulator, the working principle of the riboregulator is given in Figure 3.17C. The riboregulators were used to prevent any leakage from the uninduced cells, preventing their contribution to the curli network of the biofilm. The riboregulator enables the control of the expression of the target gene at the transcript level [196].

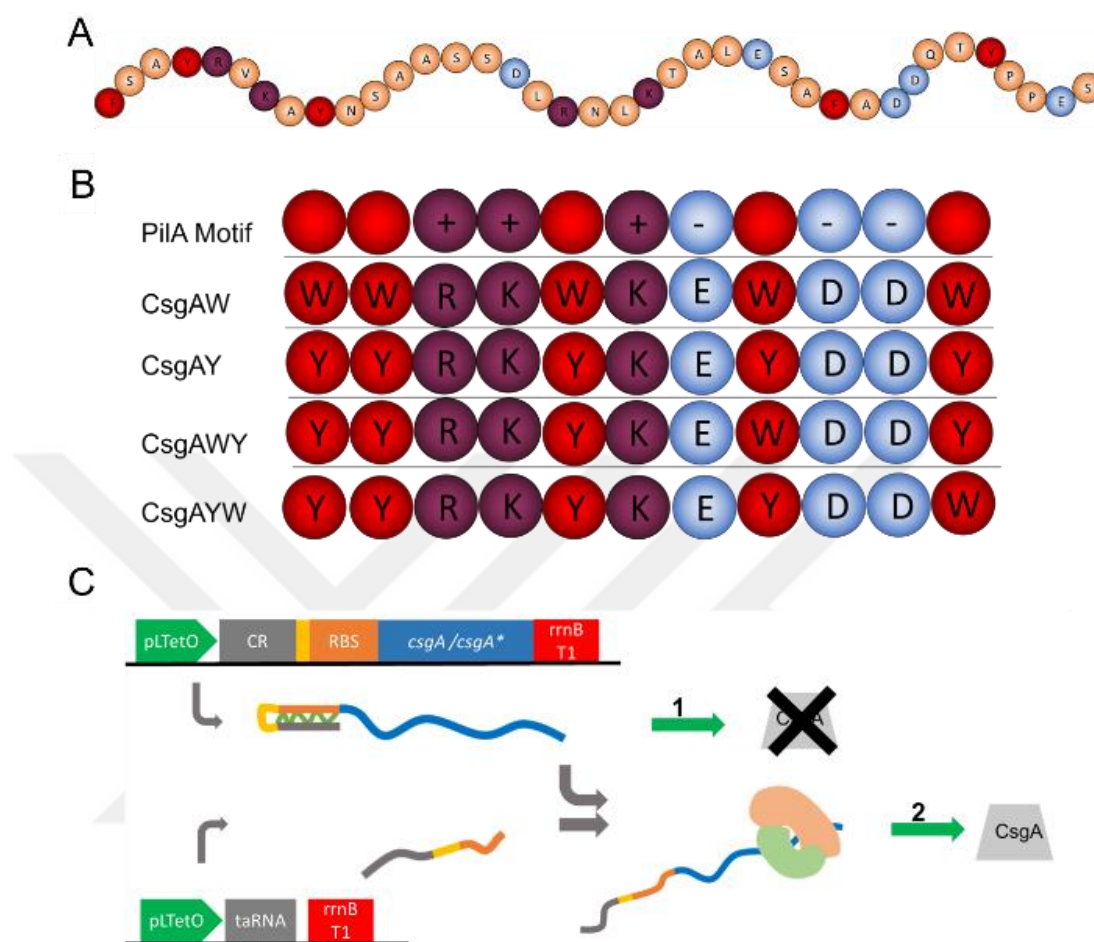


Figure 3.17: Genetic engineering of curli subunits used to form conductive nanofibers. (A) C-terminal amino acid sequence of *G. sulfurreducens* PilA protein. Orange balls indicate non-charged, non-aromatic amino acids. (B) Addition of conductivity-enhancing and aromatic amino acids to the *E. coli* CsgA protein sequence, mimicking the motifs found in *G. sulfurreducens* PilA protein. The conductive motif is composed of 5 aromatic amino acids (red), 3 positively charged amino acids (purple) and 3 negatively charged amino acids (blue). CsgAW, CsgAY, CsgAWY, and CsgAYW versions of conductive motifs are designed and fused to the C-terminal of csgA. The rationale behind this design is that aromatic amino acids are well-recognized to be important in electron transfer,

while recent studies have also demonstrated the importance of charged amino acids for electron transfer in PilA conductivity [138, 195]. Consequently, we decided to exclude linker amino acids and used a combination of aromatic and charged aminoacids in adjacent position. (C) Genetic elements of the translational riboregulator system used for the strict control of the CsgA and conductive motifs fused to the CsgA monomer. Translational riboregulator (cis-repressor (CR)) is a ribonucleic acid (RNA) that binds to the ribosomal binding site and blocks translation. taRNA is a trans-activator RNA which binds to CR and opens the ribosome binding site to allow the translation of the fusion protein. The system is controlled at the transcription level.

In order to verify that all conductive curli subunits formed thick, adherent biofilms in the presence of aTc, crystal violet biofilm assay was performed in triplicate. Biofilm formation of all the constructs was confirmed by crystal violet staining (Figure 3.6). Designed conductive curli subunits enable bacteria to attach to surfaces and can readily be observed in 24-well plates. In contrast, negative controls (*E. coli* MG1655 *PRO ompR234 ΔcsgBA* and empty pZA vector-transformed *ΔcsgBA* cells) show little attachment on the surface of 24-well plates. The formation of curli fibrils produced by recombinant bacteria were also characterized by Congo red (CR) assay. The absorbance of 25 µg/ml CR was subtracted from the absorbance of supernatant at 480nm and normalized against OD₆₀₀. The amount of CR-bound cells and curli fibers were calculated as $-\text{OD}_{480}/\text{OD}_{600}$ and represented in Figure 3.7B. Congo red assay results demonstrated that C-terminal modified CsgA variants retain their ability to form amyloid fibrils and bind ~1.33-fold more Congo red than unmodified CsgA

protein (Figure 3.7B). CR staining results also suggest that there might be a slight change in the final protein folding of CsgA, which may have an effect on the structure of the final fiber assemblies.

To acquire an idea about the morphology of curli fibers produced by recombinant cells, biofilms were analyzed with scanning electron microscopy (SEM). In Figure 3.8, SEM images reflect the assembly and morphology of conductive fibers around the engineered bacteria on silica surfaces. SEM data showed that conductive fibers were 2-2.5 μm in length and 25-35 nm in thickness. This length is consistent with 5 to 7 individual *curli* fibers coming together to form fiber bundles through hydrophobic interactions between aromatic amino acids [197].

Synthetic conductive curli biofilms were further investigated by probe station analysis. Biofilm conductivity of *Geobacter sulfurreducens* were measured in liquid medium using microbial fuel cell [198] whereas our biofilm conductivity of engineered *E.coli* were measured using dried samples on interdigitated gold electrode. Figure 3.10 shows the I/V curve of the engineered biofilms. Conductivity of CsgAW, CsgAY, CsgAWY, CsgAYW and CsgA protein expressing cells, as well as *csgBA* KO cells were compared for the determination of optimally conducting fiber motifs. Conductivity curves revealed that biofilms exhibit a linear ohmic behavior (Figure 3.10). CsgAW, CsgAY, CsgAWY and CsgAYW motifs exhibited conductivity values about 7.61, 8.13, 5.86, and 3.27 fold higher than *E.coli* ΔcsgBA cells and 4.08, 4.37, 3.14, and 1.76 fold higher than unmodified CsgA biofilms, respectively (Figure 3.10). The increase in the conductivity of synthetic curli fibers compared to native curli fibers is attributable to the conductive motifs that are fused to CsgA monomers, as the expression

systems of native CsgA and synthetic CsgAs are the same. However, differences were observed between the abilities of aromatic amino acids to influence conductive properties in synthetic peptide networks and curli fibers, due to differences in expression systems and self-assembly behavior. Nevertheless, it should be noted that the optimization of aromatic amino acid sequences through peptide systems was the rationale behind the use of tryptophan and tyrosine residues in the initial design of conductive curli fibers. According to a recent study, aromatic amino acid substitution into hepta-alanine peptides increases the conductance of the peptide chain [140], which is also observed in our engineered csgA proteins. Substitution of tryrosine with tryptophan in CsgWY and CsgAYW slightly decreases the conductivity of the proteins. Conductivity is observed as CsgWY > CsgAYW (Fig. 3.10) and their tryptophan positions can be seen in figure 3.17.

To gain insights into the conductivity of engineered CsgA proteins containing conductive peptide motifs, template-based tertiary structure modelling was performed using RaptorX, a web-based server, to obtain their 3D structure, because homology modelling is not available to model CsgA protein (Figure 3.14) [197, 199]. 3D structure of conductive CsgA obtained from RaptorX server is consistent with previous models of CsgA.

According to the predicted structures, a coil motif has been observed between 133th-135th residues, whereas the residues between 136th-141th amino acids were predicted to fold as a beta-sheet structure on top of the last beta-sheet turn of CsgA protein in all engineered CsgA models where glycine residue after the periplasmic targeting sequence of CsgA is taken as first residue. The 136th and

137th residues are assigned as the most disordered structures in the model. Interestingly, the solvent accessibility of lysines on 137th shows similar trend with conductance of engineered CsgA proteins. Due to non-aromatic nature of lysine amino acid side chains, it is not likely to participate directly the electron movement through bundle structure yet it might strengthen the lateral structure of CsgA fibers.

The conductance of proteinaceous nanowires, such as the pili of *G. sulfurreducens*, is explained by two different hypotheses, π - π stacking of aromatic amino acids and electron hopping [200, 201]. π - π stacking requires the tight packing of aromatic amino acids within 3.2 Å distances to establish a metal-like conductivity profile similar to that seen in conductive polymers with delocalized π electrons, such as polyacetylene [200]. While CsgA fibrils were previously proposed to grow in perpendicularly oriented β -strands [197], our TEM results (Figure 3.9) have revealed fiber bundles that are much thicker than single CsgA monomers (~3-4 nm for single CsgA fibers [188] against ~15-20 nm in TEM images). Consequently, CsgA fibrils appear to interact with each other laterally to form thicker bundles, and even under optimal packing, recombinant CsgA proteins are not expected to establish long-range π - π stacking interactions. In contrast, electron hopping is a more likely scenario due to the presence of tyrosine amino acids, which have oxidizable phenol side chains [202]. We therefore suggest that both short-range π - π interactions and electron hopping between stacks may be responsible for electron transfer.

3.5. Conclusion

Conductive self-assembled peptide systems are of considerable interest because of the well-recognized ability of the self-assembly process to enhance the conductivity of polymers [203, 204], and the promising applications of conductive self-assembled peptides for a range of disciplines [192, 193, 205]. In this work, we report the first demonstration of a curli-based self-assembling system that is secreted directly by *E. coli* cells and exhibits higher conductivity compared to native biofilms. Sequence optimization of the PilA-inspired peptide molecule revealed that aromatic amino acids were responsible for conductivity, possibly due to π - π stacks and electron hopping. Polytyrosine and polytryptophan motifs were strongly capable of promoting biofilm conductivity; however, mixed tyrosine-tryptophan sequences were less effective for this purpose. This phenomenon may derive from the differential pH responses associated with these amino acids, which may prevent their combination from conducting effectively in the biofilm environment. Nevertheless, monotypic repeats of aromatic amino acids are shown to impart a strong conductivity when incorporated into a CsgA-derived, self-assembling peptide sequence, and may be further optimized for various applications in energy and materials design.

3.6. Materials and Experimental Section

3.6.1. Peptide Assembly into Fibers

Peptide monomers of self-assembled fiber networks were synthesized using Fmoc and Boc chemistry. The designed peptides were synthesized (LifeTein, USA) with the addition of homotripeptide containing aromatic amino acids to the C- terminal

end of the scaffold peptides in Table 3.1. Peptide solutions in group 1 were prepared by either dissolving the pellet directly in ddH₂O, in group 2 dissolving the peptide pellet in DMSO and diluting the peptide solution in ddH₂O at a concentration of 4.0 mg/ml (for highly hydrophobic peptides), or in group 3 dissolving the pellet in DMF and diluting in ddH₂O (for tryptophan-containing peptides). The self-assembly of the peptides was performed by placing the peptide solution in a vial tube for three weeks at 27 °C for the maturation of amyloid-like fibrils as suggested previous work [192].

3.6.2. AFM Characterization of Peptides

20 µl of the peptide solutions were dropped on mica surfaces and left for overnight drying. Samples were characterized in tapping mode using Si probes with a nominal spring constant of 40 N/m and a resonance frequency of 300 kHz (TED PELLA INC, USA).

3.6.3. SEM Characterization of Peptides

10 µl of the peptide solutions were deposited on silica surfaces. After evaporation, samples were sputter-coated with 5 nm of Au/Pd alloy using a precision coating system (Gatan Inc, USA).

3.6.4. Preparation of Gold Electrodes

Interdigitated gold electrodes were prepared for the measurement of conductivity. In order to create the electrodes represented in figure 3A, we used a custom shadowing mask with 175 µm holes. All preparation was performed in a clean room environment. Thermanox coverslips were placed on glass slides and shadowing masks were placed on top of the coverslips. Shadowing masks were

adhered to coverslip surfaces with small thermal sticky tapes. 8 gold electrodes can be prepared per run of thermal evaporator. 0.5 g of gold was used per run and gold electrodes were obtained with a thickness of ~50 nm.

3.6.5. Conductivity Measurement of Peptides

For the conductivity measurement, 20 μ l of mature fibrils were drop-casted on interdigitated electrodes and left for drying in a home-made high-humidity environment. Measurements were obtained from three electrodes for each peptide assembly. Current-voltage (I-V) curves were obtained from -5 to +5 volts under a two-electrode configuration.

3.6.6. Cloning of the CsgA Conductive Peptides

csgAW, csgAY, csgAWY and csgAYW gene fragments listed in Table A.2 were amplified from pZa-tetO-csgA-CmR (primers were listed in Table B.2). Recombinant genes were cloned into pZa-tetO-CmR using the cut ligate method using kpnI/mluI restriction sites and the plasmid constructs are represented in Figure C.6, A.7, A.8, and A.9 and sequence alignments of the constructs are shown in Figure D.6, A.7, A.8, and A.9.

3.6.7. Strain and Expression of Curli Proteins

Our inducible synthetic circuits were transformed on an *E. coli* MG1655 *PRO* Δ csgBA ompR234 host strain. This strain has higher expression of curli genes from native curli operon at 30 °C in growth medium [206, 207]. In order to produce curli proteins, starting cultures were inoculated from a single colony and grown in LB medium at 37 °C for 16 h. For the experimental culture, 20 μ l cell volume from overnight cultures were transferred to 2 ml M63 minimal medium

supplemented with 1 mM MgSO₄ and 0.2 % glucose and grown at 30 °C in 24 well plates without shaking for 3 days. For the induction of biofilm formation, aTc was used in a concentration of 200 ng/μl.

3.6.8. Crystal Violet Assay

E. coli MG1655 *PRO ompR234 ΔcsgBA* and empty pZA vector transformed *ΔcsgBA* cells were used as negative control strains. For crystal violet staining, 24 well plates were stained with 400 μl of 0.1 % crystal violet (Sigma) and incubated for 10 minutes at room temperature. After staining, wells were washed three times with 1 mL ddH₂O. Images were taken using a ChemiDoc MP imaging system (BioRad).

3.6.9. Congo Red Assay

A single colonies was inoculated into 10 mL LB including relevant antibiotic and grown at 37 °C for 16 h. Then, 0.5 mL overnight culture were inoculated into 15 mL LB. When the cultures reach OD₆₀₀ between 0.3 and 0.5, aTc (final concentration of 200 ng/μl) were added to induce fibril formation for 18 hours. 1ml of cell culture were used for OD₆₀₀ cell normalization and 800 μl of cell culture were mixed with 5x Congo red solution for a final concentration of 25 μg/ml and incubated 30 min at room temperature. The cells and curli were centrifuged down at 14000 rpm for 5 min and supernatants were taken for quantification of CR. The absorbance of CR in supernatant were measured at 480 nm.

3.6.10. SEM Characterization of Biofilms

For sample preparation, biofilm formation was induced by aTc under static culture conditions. Samples were washed with ddH₂O and fixed with 2.5 % glutaraldehyde overnight at +4 °C, following by dehydration steps with increasing ethanol concentrations, followed by sputtering 5nm Au/Pd alloy.

3.6.11. TEM Characterization of Biofilms

Production of the curli biofilms were performed in 24 well plate using minimal medium supplemented with aTc (200 ng/μl), 1 mM MgSO₄ and 0.2 % glucose without shaking for 3 days 10 ul of sample were placed on a TEM grid (Ni grid with formvar/C support, 300 mesh) and incubated for 30 seconds. Then the fluid were removed from the sample and three rounds of wash with distilled water were performed on the grid for 30 seconds. Samples were incubated with negative stain dye (3% uranyl acetate) for 30 sec. and excess fluid was removed from the grid. Samples were analyzed by FEI Tecnai TEM at 200 keV.

3.6.12. Conductivity Measurement of Biofilms

Biofilms were formed in M63 medium without shaking for 3 days following induction with aTc. Biofilms were then washed twice with ddH₂O to remove medium salts and drop-casted on interdigitated gold electrodes to measure the conductivity of biofilm.



CHAPTER 4

Characterization of Mechanical and Morphological Properties of Curli Nanofibers

This work is partially described in the following publication[208]:

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4.1. Objective

Extracellular polymeric substances consist of molecules, DNAs, carbohydrates, and proteins that are secreted by microbial biofilms. These molecules assist in the synthesis of bacterial biofilms as highly ordered, complex and dynamic material systems, contribute to the adaptation of cells to their environment, and increase their flexibility and functionality under a broad range of conditions. These qualities are ideal for the design and production of next-generation biomaterials. In this study, the morphological and mechanical properties (in terms of Young's moduli) of amyloid fibers produced by genetically engineered *Escherichia coli* were examined by atomic force microscopy. Biochemical properties of the major subunit CsgA and the minor subunit CsgB were tuned by using mutant versions of curli subunits (synthesized through the addition of tag sequences) and different combination of CsgA and CsgB. Results obtained from AFM imaging and force spectroscopy showed that Young's moduli of biofilms could be rationally tuned

for the generation of bio-nano interfaces by controlling the secretion of engineered biofilm components.

4.2. Introduction

4.2.1. Self-assembled Protein Nanostructures

Self-assembly is the process through which small, typically organic components or *monomers* spontaneously form well-ordered, complex networks through non-covalent interactions. Protein nanostructures, such as viral capsid proteins [209, 210], cage proteins [211, 212], bacterial micro-compartments [213], filamentous bacteriophage coats [214], bacterial flagella [215], bacterial pili [195] and amyloids [216], are often formed through self-assembly. Amyloid systems are exceptionally resistant to the extremes of pH and temperature, as well as proteolytic degradation by enzymes, which makes them appealing for biomimetic applications [217]. Two types of amyloids exist in nature: pathological amyloids [218] and functional amyloids [219]. Functional amyloids are produced by bacteria to serve as biofilm components [220], while pathological amyloids are proteins that aggregate under disease conditions and impede with cell and tissue function [221]. Functional amyloids serve as core components of biofilms and contribute to its structural order through the formation of amyloid fibril networks [222]. While biofilms are undesirable in many settings due to their ability to increase the resilience of pathological bacteria, functional amyloids involved in biofilm production are promising for the production of highly durable and flexible next-generation biomaterials. [223, 224]. However, biofilm-based biomimetics approaches are yet to realize their full potential with respect to the production of

novel material systems. One reason for this effect is that the mechanical, chemical and biological properties of amyloid fibers are not sufficiently characterized for their effective replication in artificial systems [115]. Nevertheless, the sequences associated with amyloid fibers can be incorporated into peptide materials to replicate some of the functions associated with amyloids in easy-to-produce self-assembled systems for a broad range of applications, including regenerative medicine and drug delivery [114, 225-227].

4.2.2. Tunable Properties of Bacterial Biofilm

Biofilms are produced ubiquitously in the bacterial kingdom, including the archetypical model bacterium *Escherichia coli*. In *E. coli*, biofilm formation is primarily mediated by two proteins, CsgA and CsgB, which are regulated by the *curli* operon. Curli is activated under environmental stress and induces the bacterial cell to switch between its mobile planktonic phase to stationary biofilm phase. This transition is accompanied by the production of a biofilm backbone by CsgA and CsgB, which are extruded from the cell membrane through the action of three other proteins in CsgE, CsgF and CsgG [222]. CsgB acts as a nucleating agent and stimulates the assembly of CsgA, which is responsible for the formation of the biofilm network itself [228]. Due to its simplicity and versatility, this secretion process is often altered for bioengineering approaches. For example, the controllable release of CsgA networks has recently been demonstrated in *E. coli* [223], and the CsgA protein was later used to form a chimera with mussel foot proteins to produce an easy-to-produce, water-compatible adhesive material [227]. As such, curli proteins can be used as an effective expression system for the design and synthesis of next-generation biomaterials.

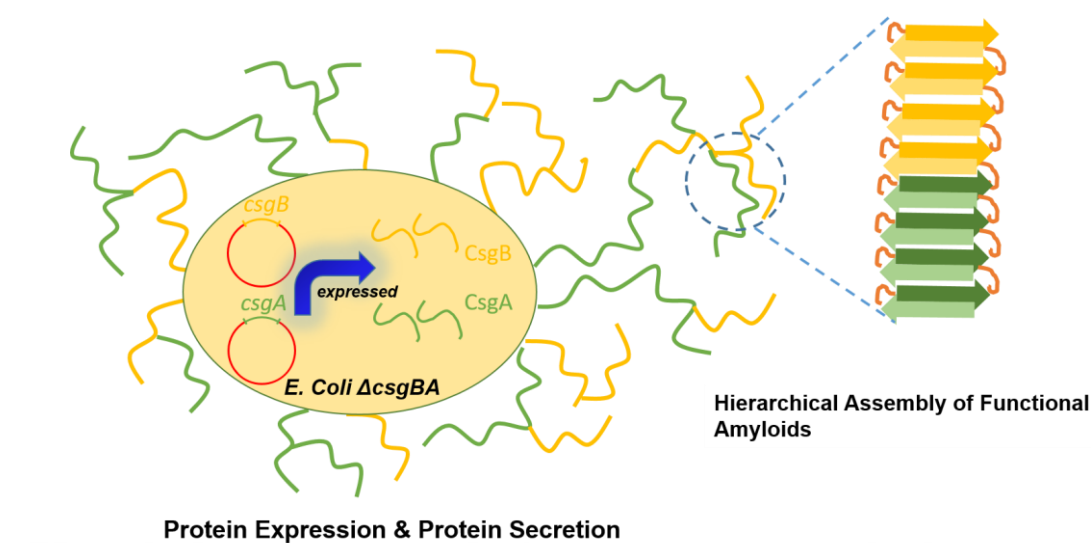


Figure 4.1: Expression, assembly and secretion of curli proteins in *E.coli*.

4.2.3. Atomic Force Microscopy for Biological Materials

Although biofilm expression systems have been used successfully in a number of bioengineering studies, their relative lack of characterization limits their potential in practical applications. In particular, mechanical properties of biofilm components are especially important for their utility in biomedicine, but remain incompletely characterized. Atomic force microscopy (AFM) is the premier technique for the mechanical characterization of biological materials, and distinguishes itself from other imaging techniques through its compatibility with liquid environments [229-233]. As biomaterials are liable to changing their elastic moduli depending on environmental conditions, samples imaged in air do not accurately represent their mechanical properties, and the ability of AFM to be operated in aqueous systems is critical for their characterization [234, 235]. AFM has been used for the high-resolution imaging of a wide variety of biological samples, including cells [236, 237], single proteins [238], genetic material such as chromosomes, chromatin and DNA [239], and cell membranes [240]. In addition,

force-distance (FD) analyses have been used to show that cancer cells exhibit a different elastic modulus compared to their healthy counterparts [241]. FD analysis has also been employed to evaluate the effect of antibiotic treatment on the integrity of bacterial cell walls [242] and to determine the elastic modulus of various cell types [243]. While a high-resolution AFM map is typically acquired in 1 – 20 minutes, high-speed AFM methods [244] have also been employed for the real-time observation of biological processes [245] and their changes in response to environmental stimuli [246]. However, there is a relative dearth of information regarding the mechanical characterization of amyloid systems using AFM [247], although FD experiments have been used to investigate the mechanical properties of insulin-based amyloid fibers [248, 249]. AFM has also been used in the mechanical investigation of bacteria capable of biofilm production, but the amyloid structures found in the biofilm were not individually characterized [250, 251].

In the curli expression system, the structural integrity of the biofilm depends strongly on the CsgA and CsgB subunits that form the material structure. Consequently, the controlled expression of these components can be used to modulate the elastic modulus of the complete fiber structure, and the effect of subunit identity and concentration on biofilm properties were tested using AFM in the present study. In addition to native CsgA and CsgB sequences, modified proteins containing histidine tags were also used to determine their ability to alter amyloid fiber structure and/or Young's moduli, and the general morphology of the fiber systems were evaluated using AFM imaging. The results show that the elastic modulus of curli fibers can be altered through changes in expression

patterns and subunit sequences, which is promising for the use of curli and other biofilm components as next-generation biomaterials with tunable mechanical properties and bioactive sequences.

4.3. Results

4.3.1. Morphology of Amyloid Nanofiber Structures

Figure 4.1 shows the expression system used for the production, secretion and self-assembly of curli fibers. CsgA and CsgB exhibit different morphologies and self-assemble through different pathways; consequently, differences in their expression patterns are expected to create corresponding differences in the final fiber network. These subunit-mediated changes are best understood through the characterization of nanofibers formed by individual CsgA and CsgB subunits. In addition to their native versions, these subunits were also modified through the inclusion of a histidine linker sequence, introducing an additional layer of complexity to the expression system. This linker is predicted to induce structural alterations in both CsgA and CsgB, with CsgB being altered to a greater extent than CsgA. Consequently, linker-modified CsgB is expected to impart a greater change in elastic modulus to the curli fiber network.

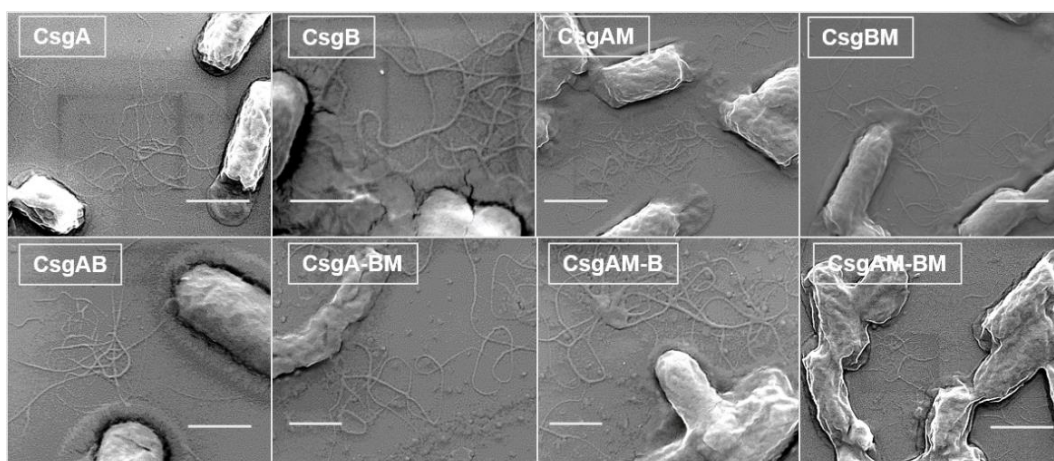


Figure 4.2: Scanning electron microscopy images of the biofilm proteins.

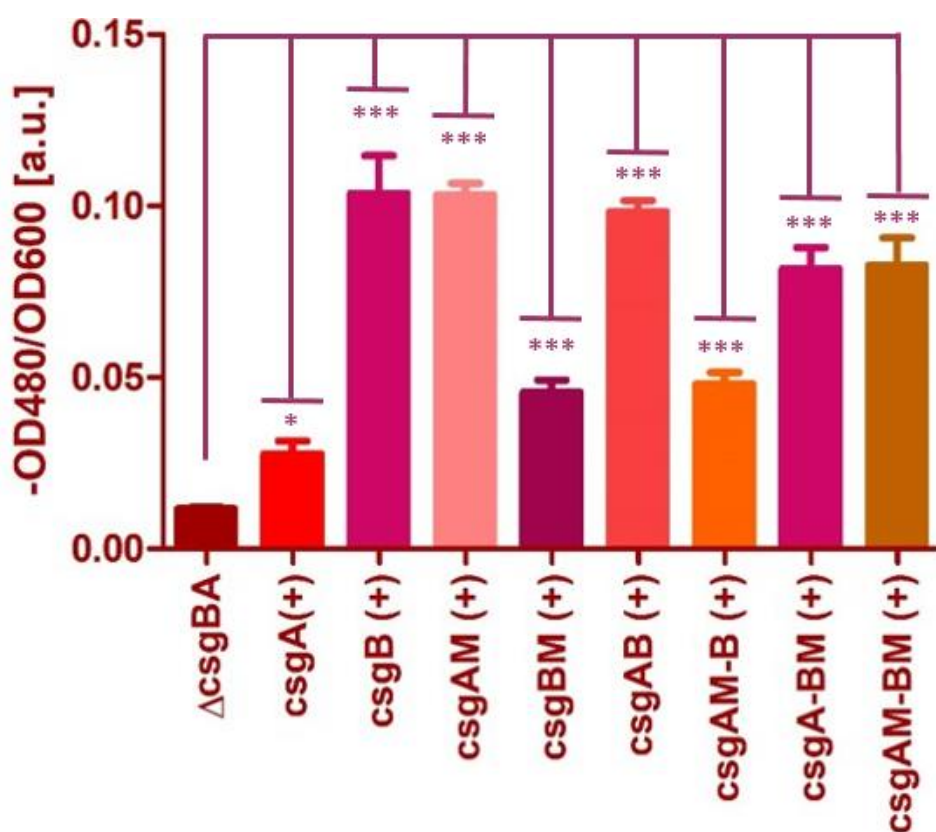


Figure 4.3: Congo red assay for biofilm proteins. One-way analysis of variance test was used to compare all samples with the control sample (*, significant; ***, extremely significant). $p < 0.05$).

The effect of nanofiber identity and histidine tag inclusion on fiber elastic moduli were tested using the complete fiber system (CsgAB), the individual fiber components (CsgA or CsgB), their histidine-tagged counterparts (CsgAM, CsgBM and CsgAM-BM), and hybrid fibers containing one modified and one unmodified element (CsgAM-B and CsgA-BM). An *E. coli* host lacking CsgA and CsgB were used to minimize potential interference posed by the native expression of these genes. All groups were designed to be secreted by the host cell and SEM images were acquired to demonstrate successful fiber secretion (Figure 4.2). Congo red staining [252] was also performed to show that all expression

systems were able to produce curli fibers, which are stained selectively by this dye (Figure 4.3). In contrast, the native *E. coli* $\Delta csgBA$ cells were incapable of secreting curli nanofibers. AFM has also been employed to characterize the general morphology of the curli fibers produced by each sample group in Figs. 4.4, 4.5 and 4.6.

Data pertaining to the un-tagged CsgA and CsgB subunits and their combination (CsgAB) are provided in Figure 4.4. While CsgA-only fibers were observed to form a limited spread zone around bacterial cells (Figure 4.4a), the CsgB-only system exhibited a porous network (Figure 4.4b), and the complete CsgAB fibers were observed to show a morphology similar to the CsgA-only network (Figure 4.4c).

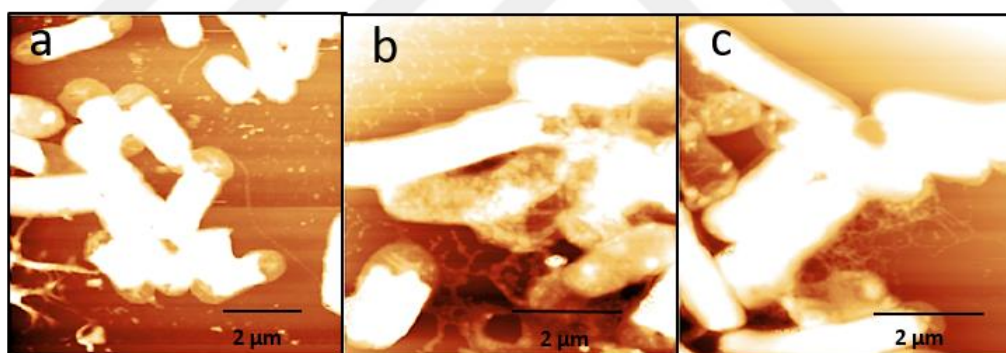


Figure 4.4: Morphology of unmodified curli subunits. a) Cells secreting a) only CsgA, b) only CsgB c) both CsgA and CsgB.

Data pertaining to the histidine-tagged CsgAM and CsgBM subunits and their combination (CsgAM-BM) are provided in Figure 4.5. CsgAM and CsgBM (Figures 4.5a and 4.5b) were observed to be broadly similar to one another and exhibited a porous morphology around the host bacteria, the CsgAM-BM system

was associated with longer curli fibers that were distributed evenly across the substrate (Figure 4.5c).

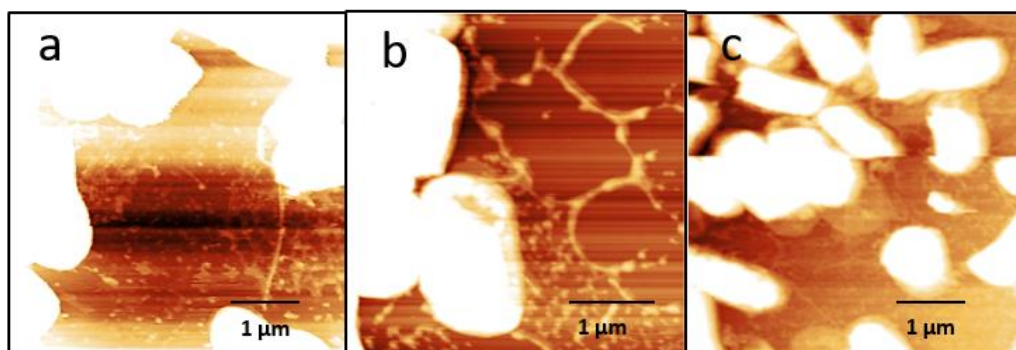


Figure 4.5: Morphology of modified curli subunits. a) Cells secreting a) only CsgAM, b) only CsgBM c) both CsgAM and CsgBM.

Data pertaining to the hybrid fibers (CsgAM-B and CsgA-BM) are provided in Figure 4.6. The CsgAM-B system was found to form an extensive and porous network over the substrate surface, while the CsgA-BM system was likewise observed to produce an extensive biofilm network around the bacterial cells, although with very little porosity.

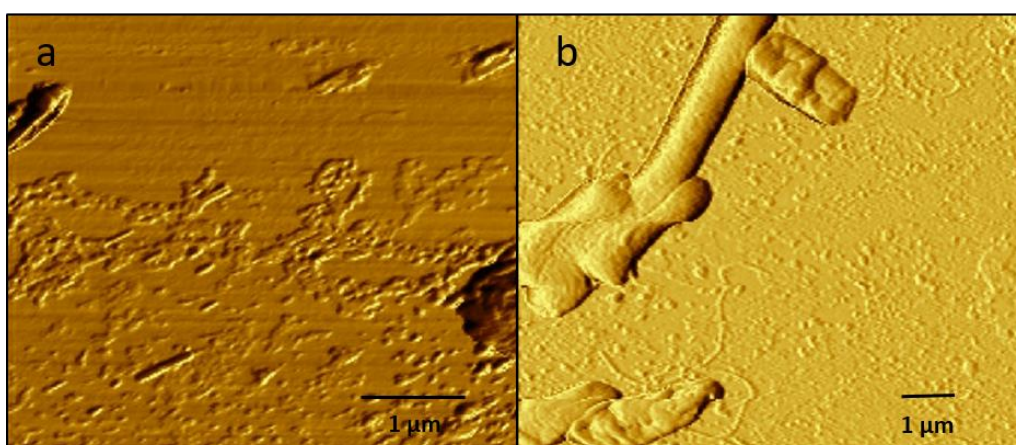


Figure 4.6: Morphology of hybrid curli fibers. a) Cells secreting a) both CsgAM and CsgB, b) both CsgBM and CsgA.

The height profiles of each nanofiber group were also measured by AFM as follows: CsgA (60 nm), CsgB (13.5 nm), CsgAB (9 nm), CsgAM (5 nm), CsgBM (12.5 nm), CsgAM-BM (17.5 nm), CsgAM-B (12.5 nm), and CsgA-BM (7.5 nm). However, no strong associations between fiber height and sample type could be determined.

4.3.2. Tunable Young's Modulus of Amyloid Nanofiber Structures

Following morphological characterization of curli fibers formed by native and histidine-tagged CsgA and CsgB components, force-distance analyses were performed to determine the effect of subunit type and histidine modification on fiber elastic moduli. Young's modulus histograms associated with each sample group are summarized Figure 4.7. Mechanical properties of amyloid nanofibers are clearly influenced by their components, and the elastic modulus of composite fiber systems is invariably higher than their individual subunits. Accordingly, the Young's modulus of CsgAB (11.6 MPa) is higher than the E_{mean} value for both CsgA (9.5 MPa) and CsgB (10.0 MPa), while a similar trend holds for CsgAM-BM (14.8 MPa vs. 8.4 MPa and 13.9 MPa), CsgAM-B (10.4 MPa vs. 8.4 MPa and 10.0 MPa) and most significantly, for CsgA-BM (18.9 MPa vs. 9.5 MPa and 13.9 MPa). Consequently, the curli nanofiber system is mechanically tunable through the modification of its individual subunits.

In addition, the relationship in the elastic moduli of non-modified CsgA, CsgB and CsgAB systems is $E(\text{CsgAB}) > E(\text{CsgB}) > E(\text{CsgA})$, and a similar trend of $E(\text{CsgAM-BM}) > E(\text{CsgBM}) > E(\text{CsgAM})$ is observed for the histidine-tagged subunits. Consequently, the inclusion of the CsgB subunit appears to impart an

additional measure of stability to the fiber system, increasing its tolerance to mechanical stress and consequently its elastic modulus values.

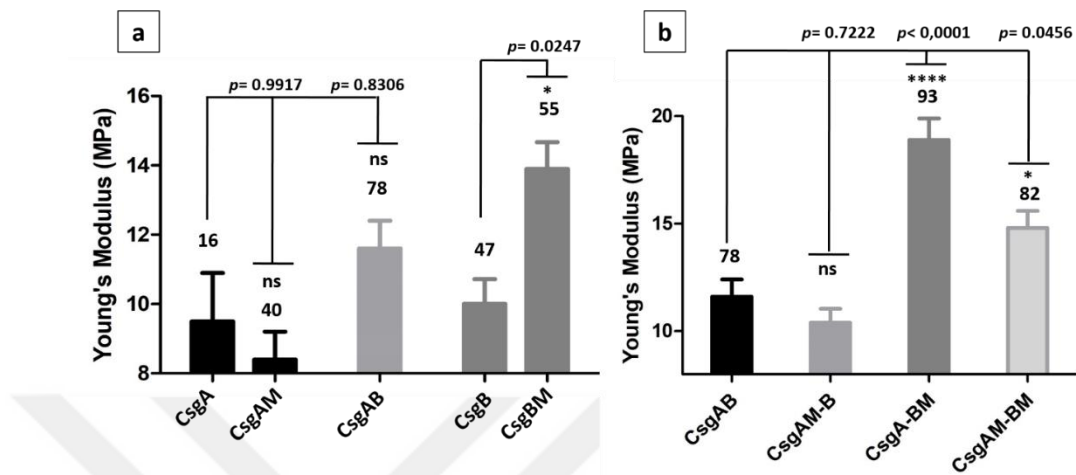


Figure 4.7: Young Modulus's comparison of single curli fibers in (a) and mixture of major and minor curli fibers in (b). Number on each column represents the repeated measurements. Error bars are standard error of mean. For the statistical analysis two-way anova was used for single curli fibers (a) and one-way anova was used for mixtures of major and minor curli fibers (b). Young modulus increases only with the addition of linker molecule to CsgB not CsgA. (ns, not significant; *, significant;****, extremely significant; $p < 0.05$)

4.4. Discussion

AFM analyses of fiber morphology and elastic moduli demonstrate that CsgA and CsgB expression and sequence can substantially alter the mechanical properties of curli nanofibers. Individual expression of CsgA and CsgB proteins was previously shown to affect the final morphology of curli fibers through Congo red staining, with CsgB acting as a nucleator for the self-assembly of CsgA [253]. However, no significant differences were observed between the elastic moduli of CsgA, CsgB and CsgAB systems, suggesting that these morphological changes are not fully

reflected in the mechanical resistance of the fibers. As the sequences and assembly mechanics of CsgA and CsgB are strongly similar, this lack of difference in elastic moduli is perhaps unsurprising. In addition, it is interesting that the native curli nanofiber (CsgAB) does not exhibit a significantly different elastic modulus compared to its components in CsgA and CsgB.

The effect of a histidine linker sequence on the mechanical properties of CsgA and CsgB subunits was also investigated using AFM analysis. Histidine tag inclusion was found not to significantly change the elastic modulus of CsgA; however, the CsgB sequence exhibited a significantly higher elastic modulus following the attachment of the linker sequence, suggesting that minor alterations in CsgB sequence can strongly alter its assembly mechanisms and mechanical integrity. The inclusion of this modified CsgBM subunit into the complete curli structure (*i.e.* formation of the CsgA-BM system) resulted in a drastic increase in elastic moduli, while the inclusion of the histidine-tagged CsgAM subunit (*i.e.* the CsgAM-B system) did not exhibit a significantly different elastic modulus compared to the CsgAB baseline, suggesting that the CsgB subunit is a crucial determinant of the Young's modulus of the overall system. This observation is consistent with the previous reports concerning the role of CsgB as a nucleating agent of CsgA, as well as an amyloid-forming protein in itself [254, 255]. Consequently, the modification of the CsgB protein appears to be a suitable means of altering the elastic modulus of the curli biofilm system. However, the CsgBM molecule is incapable of producing stable biofilm systems by itself, and the major curli subunit CsgA is necessary for the formation of extensive biofilm networks rather than porous structures (Figure 4.5c, 4.6b).

Lastly, a note should be made of the Young's moduli described for amyloid nanofibers in this work, which are in the MPa range. While this value is generally supported by other studies^{43-44, 55}, many other reports suggest that the elastic modulus of amyloid fibers are in the GPa range, as measured by AFM and other nanoindentation systems.^{45, 56-59} This discrepancy is attributed to differences in the fine structural properties of amyloid fiber structures; as these studies that report Young's moduli in the GPa range usually work with defect-free, straight, tubular structures with well-defined diameters and packing schemes [256-260]. In contrast, studies that report MPa-range elastic moduli for amyloid fibers do not use such "ideal" amyloid systems, instead employing more chaotic models with defects, branching structures and irregular arrangements [183, 261, 262]. The effect of fiber uniformity on mechanical stiffness is best demonstrated on two independent studies that both concern insulin amyloid fibers, with the study using more regular structures reporting a higher Young's modulus (~3 GPa) compared to the study with less well-defined structures (which have an elastic modulus of ~5-50 MPa) [256, 262]. As such, the complex amyloid network formed by bacterial cells in the present study is expected to have a lower Young's modulus values, suggesting that the MPa-range elastic modulus values are reliable. In addition, curli fibers are subject to local defects and destabilizations that decrease the mechanical stability of the system [183], which may further contribute to the lower Young's moduli observed in the present study.

4.5. Conclusion

Biofilms mediate the expansionist growth of bacterial colonies and can maintain optimal conditions for development in hostile environments by coordinating the

actions of individual cells. Functional amyloid fibers form the basis of the biofilm structure in *E. coli*, and CsgA and CsgB proteins are responsible for nanofiber formation. These proteins can be reprogrammed by genetic engineering for biomedical and biotechnological applications, as they are capable of hierarchical self-assembly. However, the mechanical and biochemical characterization of these proteins is necessary to make full use of their structural and functional properties. The control of cellular behavior by mechanical signals in particular has recently attracted great interest, and the development of materials with adjustable mechanical properties will no doubt lead to the development of new biomaterials and structures.

The formation and growth of nanomaterials on engineered nanofiber structures produced by the biofilm bacteria has previously been demonstrated [223, 224]. Similarly, hybrid materials have been produced through protein-based nano-assemblies [294][263], where biofilm proteins serve as a new template for producing nanofibers with different properties. In this chapter, our aim was to explain how the mechanical stiffness of amyloid nanofibers can be adjusted. The results showed that (i) the linker-modified CsgB protein has an important role in controlling the mechanical properties of the amyloid nanofiber structures and (ii) the CsgA protein and its linker-modified version assists in the formation of continuous, large-scale amyloid nanofiber assemblies. In conclusion, controlling and changing the mechanical properties of functional amyloid nanofibers is promising for the development of novel scaffolds in tissue engineering and nanowire structures for new applications in materials science and nanotechnology.

4.6. Materials and Experimental Section

4.6.1. Preparation of Genetic Constructs and Cloning of *csgA* and *csgB*

Gene fragments were amplified from genomic DNA of *Escherichia coli* DH5a. *csgA* gene fragments were amplified by using the primers listed in Table B.3 in the Appendix. F1-R1 Primers were used to amplify CsgA and F2-R2 primers were used to amplify CsgB; their sequences are listed in Table B.3 in the Appendix. Recombinant gene fragments were cloned into pZa-CmR-aTc-ribo vector and pZa-AmR-aTc-ribo vector with cut ligate method by using *kpnI* and *mluI* endonucleases (NEB). Recombinant plasmids were isolated with QIAprep Spin Miniprep Kit. Plasmid constructs were sequence-verified by Genescript. In addition, *csgA* fragments amplified from *Escherichia coli* DH5a genome and soft Linker sequence (3xGGGS), TEV protease restriction site and soft Linker sequence (GGGS) were added to 3' prime of the wild type *csgA* gene with two PCR reactions. Then, PCR products were cloned into pET22b (+) expression vector. *NcoI* and *XhoI* (NEB) endonucleases were used in digestion reactions and T4 ligase (NEB) enzyme was used in the ligation reaction. QIAprep Spin Miniprep Kit was used to isolate plasmids and they were verified by Genewiz. In order to clone *csgB* gene, *csgA* gene fragments were extracted from the new recombinant vector by using *NcoI* and *KpnI* (NEB) endonucleases. F3-R3 and F4-R4 primers were used to construct the modified CsgAM protein coding gene fragments and cloning steps were achieved as described above. Genes coding for CsgBM protein was prepared using F5-R5 and F6-R6 primers. The sequence

information of the primers is given on Table B.3. Plasmid maps of cloning of csgAM and csgBM is found on appendix (C10-11).

4.6.2. Biofilm Formation Using Engineered Constructs

Our inducible synthetic circuits were transformed to *E. coli* MG1655 *PRO* Δ csgBA *ompR234* host strain. csgA and csgB genes were knocked out in this strain. For the starting culture, a single colony was inoculated to LB medium at 37 °C for 16 h. For the induction of biofilm formation, anhydrotetracycline (aTc) or IPTG were added into M63 minimal medium supplemented with 1 mM MgSO₄ and %0.2 glucose, and cultures were grown at 30 °C in 24 well plates without shaking for 3 days.

4.6.3. Congo Red Staining of Curli Structures

In order to assay the formation of the functional amyloids *in situ*, a single colony was inoculated into 3 mL LB including relevant antibiotic and grown at 37 °C for 16 h. Then, 0.34 mL overnight culture was transferred into 10 mL LB. Induction of curli fibers was performed with aTc (final concentration of 200 ng/μl) or IPTG (final concentration of 1mM) for 24 hours. 900 μl of cell culture was mixed with 5X Congo red solution (final concentration of 25 μg/ml) and incubated for 30 minutes at room temperature. For the quantification of curli bound Congo red (CR), cells were centrifuged at 14000 rpm for 5 min. The absorbance of 25 μg/ml CR was subtracted from the absorbance of supernatant at 480 nm (AbsCR_{OD480}) and normalized for total cell concentration (AbsCR_{OD600}). The calculation of the

amount of CR bound cells and curli fibers was performed via the following expression: $(- \text{AbsCR}_{\text{OD480}} / \text{AbsCR}_{\text{OD600}})$.

4.6.4. SEM imaging of Curli Structures

After the formation of biofilms on 24 well plates, M63 minimal medium of the wells were removed. Cells were washed two times with phosphate-buffered saline (PBS). Then, cells were fixed on silicon wafer with 2.5 % glutaraldehyde solution in PBS overnight at +4 °C. After fixing cells, dehydration was performed via serial ethanol washing steps with increasing concentration. Samples were dried at room temperature and were sputtered with 5 nm Au/Pd alloy. FEI Quanta 200 FEG scanning electron microscopy was used for imaging.

4.6.5. AFM Imaging and Characterization of Young's moduli of the Biofilm Structures

High-resolution imaging (in contact mode) and FD experiments were conducted under ambient conditions (temperature: 21 ± 1 °C, relative humidity: $25 \pm 5\%$) using a commercial AFM instrument (Nanomagnetics Inc.). A single AFM probe (Nanosensors PPP-CONTR) with a spring constant of 0.20 N/m (determined via the Sader method [264]) has been utilized for all experiments. The geometry of the AFM probe apex has been determined via scanning electron microscopy [208]. All samples have been prepared by drop-casting on freshly-cleaved mica substrates and subsequent drying under ambient conditions. The experimental procedure associated with the use of FD curves toward the determination of Young's modulus values is explained in appendix E in detail [208]. In summary, a

series of FD curves are acquired on each sample to be investigated, where curves acquired on the cells themselves and the mica substrate are used as control experiments. Each curve is linearly fitted and the corresponding equivalent stiffness (with contributions from both the sample and the cantilever) was calculated. Indentation into the sample was determined by comparison with FD curves acquired on the hard mica substrate and subsequently, force-indentation curves were extracted. To determine the Young's moduli from these data, the Hertzian contact model was used, taking into account the flat-ended geometry of the specific tip apex employed in the experiments.

4.6.6. Molecular Modelling of the Proteins

The CsgA, CsgB proteins and their derivatives with Linker are modelled via RaptorX web-based 3D protein structure prediction tool, which generates multiple structures [199]. Among the multiple structures generated by the Raptor X algorithm, the model that has the highest score, which also coincides with the previously generated model in the literature, was selected [265].

4.6.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5. One-way and two-way ANOVA was used to compare the differences between the groups. Error bars indicate as $\pm$ SEM (standard error of mean). At least three independent repeats were performed for each *in vitro* experiment, with a minimum of $n = 16$ for each repeat.

CHAPTER 5

Genetic Logic Gate Based Biofilm Control

5.1. Objective

Our aim was to facilitate the externally controllable patterning of curli fibers using recombinase-based logic gate memory cells. By inducing two different recombinases, we sought to engineer a logic gate to activate two different curli fibers, such as CsgA and CsgA-Histaq. Using labeled CsgA enables us to keep track of the fiber component.

5.2. Introduction

Curli fimbria, also called thin aggregative fimbria, are one of the based proteinaceous material of the biofilm structure produced by *Enterobacteriaceae* [81, 266, 267]. Curli fimbriae form 6-12nm fibers which are 0.5-1 μm in length[86]. It is also reported that curli proteins are protease and detergent

resistant [82, 86]. Curli fibers are found in the extracellular matrix to be involved in adhesion to surfaces, communication between cells, and host colonization [267-271]. Structure of amyloid fibers are generally defined as ‘cross-beta spine’ in which parallel β sheets are perpendicular to the fiber axis [19, 272-274]. Curli producing cells bind congo red dye when they were grown on CR supplemented agar plates [180, 275, 276]. Curli fibers can also be detected by the amyloid specific dye Thioflavin T (ThT) [86, 277, 278]. Curli has two structural protein components [267]. CsgA protein is the major subunit, while CsgB protein attaches to cell membrane and allows CsgA to polymerize on it [181]. Periplasmic protein CsgC is the inhibitor of CsgA polymerization in the intracellular environment [92]. The transcription, secretion and extracellular translocation of structural curli proteins are controlled by the *csgDEFG* operon [96, 99, 181, 279-282]. Here, we engineered recombinase based genetic logic gates encoding AND, and OR functions to control the expression of structural protein CsgA with 6x-His tag modification using two independent control signals (Figure 5.1 and 5.2).

We used two chemical signals for the input line for the circuits. First signal is anhydrotetracycline (aTc) molecule and second signal is IPTG molecule. These signals control the synthesis of the recombinase enzymes that are the intermediate elements of the circuits. Our dual recombinase controller vector includes TP901 and Bxb1 recombinase enzymes from Bonnet *et al* [283]. We also replaced the pBAD promoter of TP901 with pLacO promoter (Figure C.12). These enzymes recognize the DNA sequences of the recombinant sites and invert the regions. In order to build the circuits, we used two vectors. One of the vectors is for the

synthesis of the recombinase enzymes (Figure C.12) and the other vector is for the synthesis of the curli subunits (Figure C.13-14).

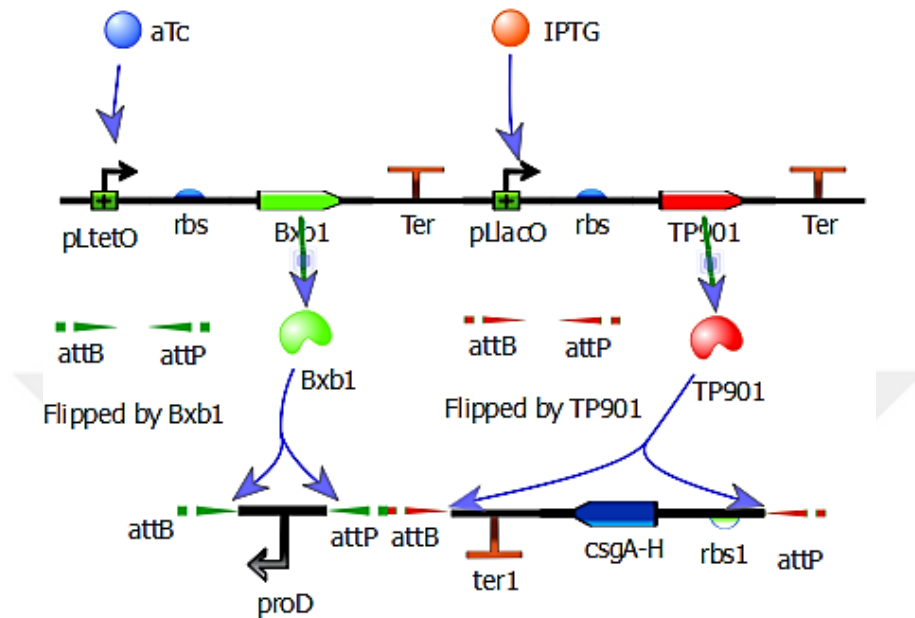


Figure 5. 1: AND GATE circuit design.

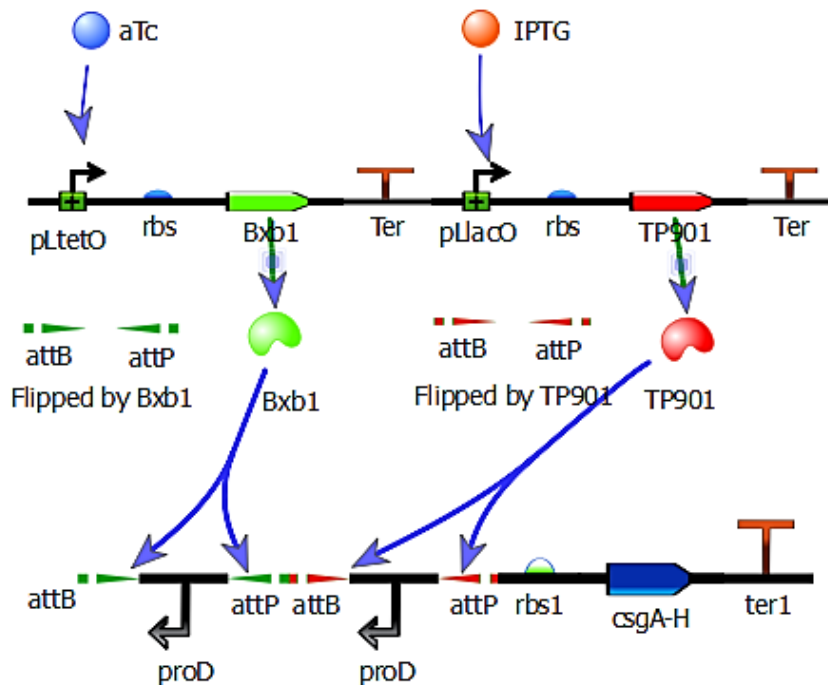


Figure 5. 2: OR GATE circuit design.

5.3. Results

5.3.1. Cloning of AND Logic Gate

The AND logic gate circuit (Figure 5.1) was artificially synthesized as a gene fragment with homologous regions to the pZa-AmpR backbone (Genewiz, USA) and cloned into the linearized pZa-AmpR vector with AatII and AvrII enzymes (Figure 5.3) using Gibson assembly method. The linearized vector was purified from agarose gel using gel extraction kit. Plasmid construct are shown in Figure C.13. Sequences were verified by sequence alignment shown in Figure D.11. The pBAD promoter of TP901 was replaced with pLlacO promoter (Figure C.12). The dual recombinase vector (Addgene Plasmid #44456) [283] and pLlacO promoter were amplified from from pZs2-123 vector (Addgene plasmid# 26598) [284] using primers in Table B.4. Preliminary confirmation of AND GATE vector was performed by digestion and visualization with AflII and KpnI enzymes. Sequence verification is also found in Figure D.11.

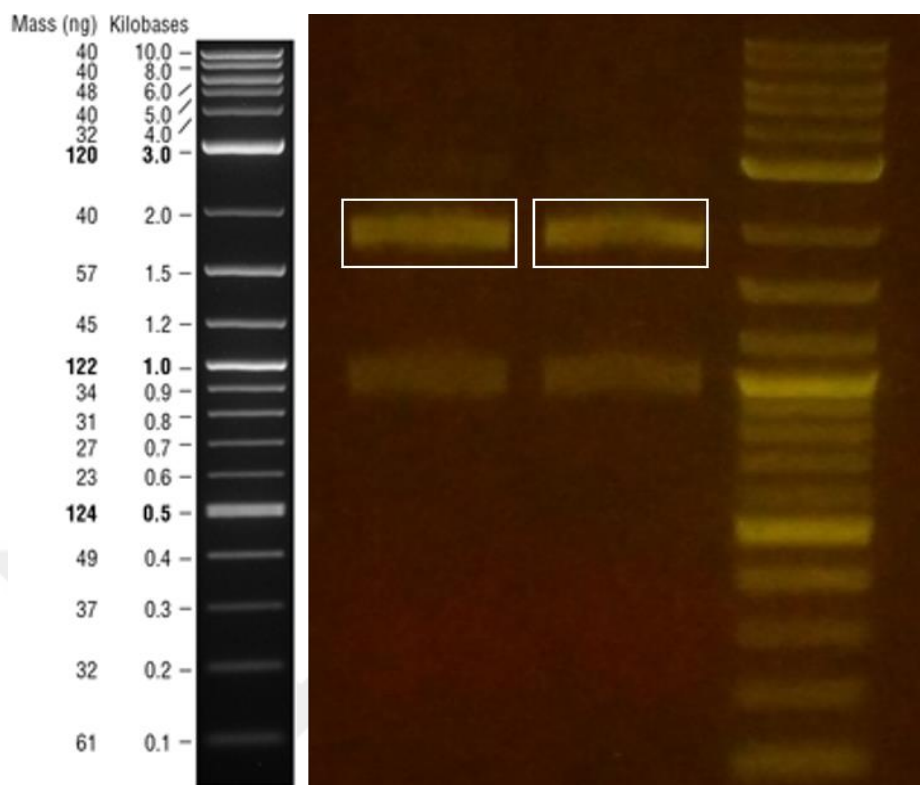


Figure 5.3: Linearized pZa AmpR backbone which contains tetO-csgB-terminator. Vector was digested with AatII and AvrII restriction enzymes. Linearized backbone is 1913bp.

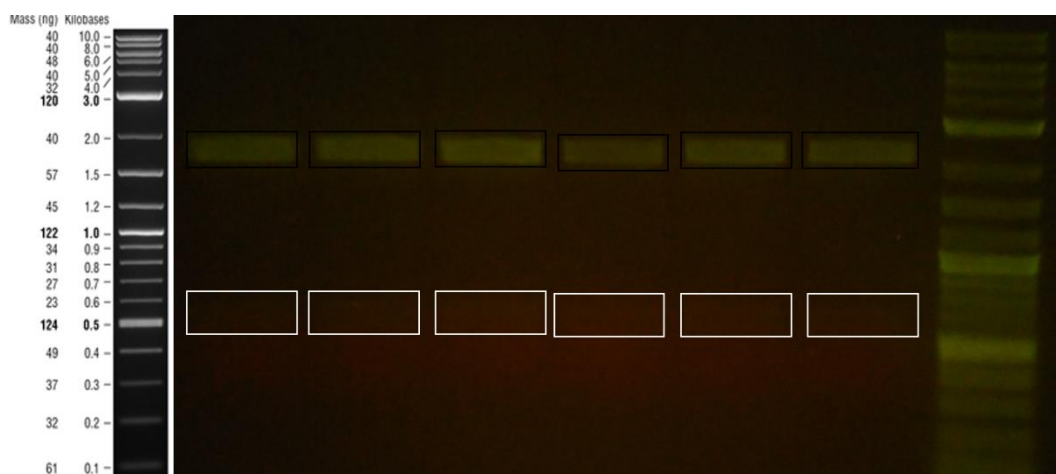


Figure 5.4: AND GATE vector following digestion with AflIII and KpnI, confirming the cloning of the AND GATE into the pZa-AmpR backbone and allowing the removal of the inverted csgAH gene fragment for the further cloning of the OR GATE. Inverted csgAH is 575bp.

5.3.2. Cloning of OR Logic Gate

To obtain the OR GATE genetic circuit (Figure 5.2) which contains two inverted proD promoters and csgAH gene, the AND GATE were digested with AflII and KpnI to replace inverted csgAH with another inverted proD promoter. Inverted proD promoter was then inserted into the sequence by PCR reaction using the AND GATE gene fragment (Figure 5.5). Its length was about 200 basepairs. The promoter and backbone were ligated using overhangs by Gibson assembly method. After cloning of second inverted proD promoter, the vector was linearized with XmaI in order to clone csgAH gene (Figure 5.6). Linearized backbone is about 2400 bp. Five colonies (from a total of six) were found to have the correct size for the linearized vector and were used for further modification. csgAH gene fragment was amplified using PCR (Figure 5.5). csgAH with overhangs is about 650 bp in length. The csgAH gene fragment and the backbone containing two inverted proD were ligated with Gibson assembly method. After cloning of the OR GATE, the length of the total circuit was confirmed by restriction digestion with AatII and KpnI enzymes (Figure 5.7). The Or gate is about 1200 bp and the backbone is about 2000 bp. A colony containing the correct insert was sequenced to verify the success of the assembly process, as shown in Figure D.12.

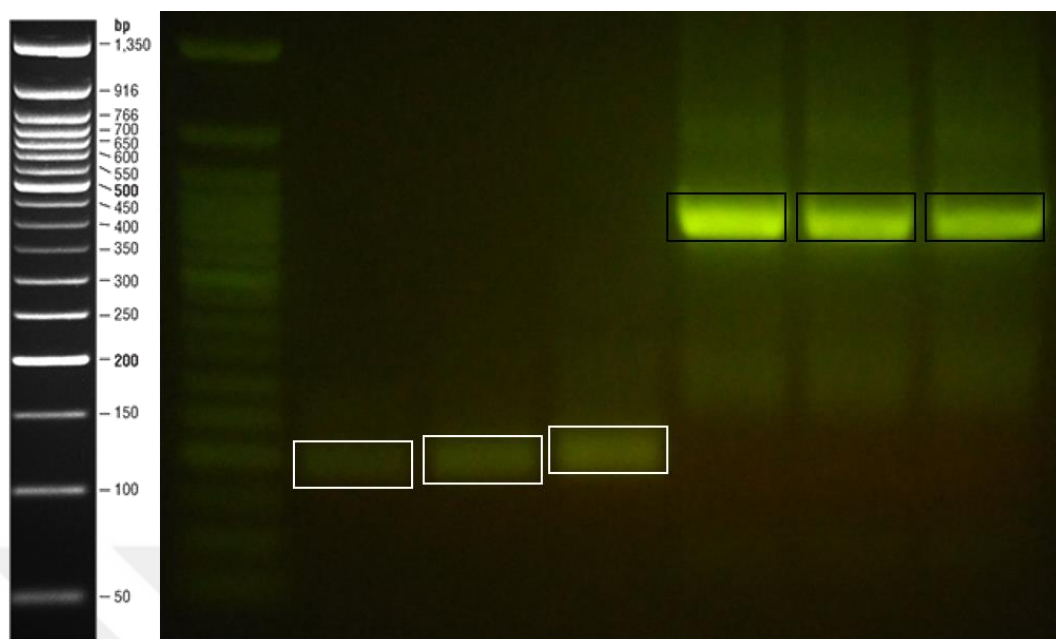


Figure 5.5: PCR results of inverted proD and csgAH. Inv proD is about 200 bp and csgAH with overhangs is about 650 bp. 50 bp ladder was used to determine the lengths of DNA fragments.



Figure 5.6: Linearized backbone for the cloning of csgAH. After the cloning of second inverted proD instead of inverted csgAH, the vector was digested with XmaI to linearize. Amplified csgAH was cloned into XmaI digested backbone which contains two inverted proD. Linearized backbone is about 2.4kb.

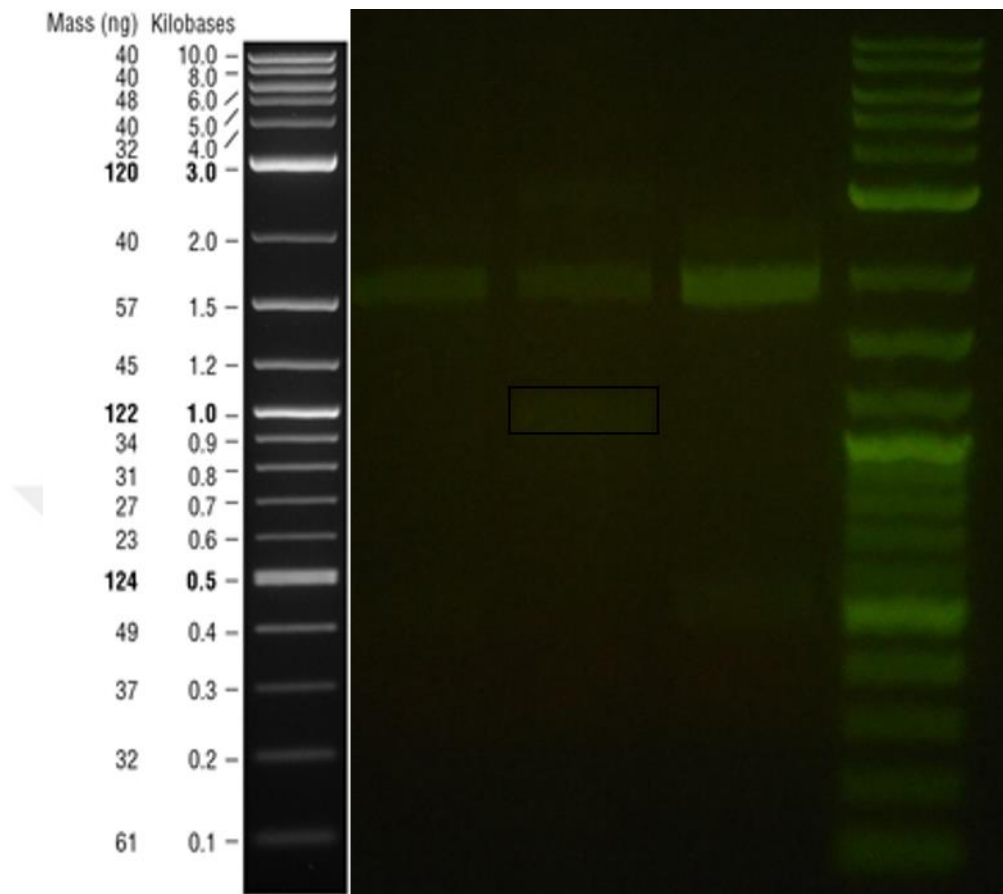


Figure 5.7: Confirmation of OR GATE cloning. The OR GATE was digested with AatII and KpnI. The OR GATE is about 1200 bp. The second colony contains the correct insert.

5.3.3. Congo Red Staining

Sequence verified AND and OR Gate plasmids (Figure C.13-14) were co-transformed with Dual Recombinase-pLtetO-Bxb1-pLlacO-TP901-CmR plasmid (Figure C.12). After co-transformation, single colonies were grown overnight in LB medium. Then they are transferred with 1/100 dilution into M63 minimal medium containing ampicillin and chloramphenicol antibiotics. aTc and IPTG were used for the induction of recombinase enzymes TP901 and Bxb1.

Recombinase enzymes were used to invert the DNA from recognition sites, as shown Figure 5.1 and Figure 5.2. Cells were incubated at 30 °C without shaking for the activation of biofilm formation.

Congo red staining performed on 6-day old cultures. Congo red is specific to curli fibers. In the 6 days of incubation, OD₆₀₀ values were measured for the normalization of congo red binding to curli fibers. Then, congo red staining of cell cultures were performed in 25 µg/ml congo red solution. Cells were centrifuged down and supernatant were measured at 480 nm for congo red quantification. Congo red binding is measured using the formula (-OD_{480/600} [a.u.]). Congo red staining shows that, for the AND GATE circuit, the expression of curli fibers occurs in high concentrations following the addition of both IPTG and aTc inducers. For the OR GATE circuit, the expression of curli fibers occurs in the presence of either IPTG or aTc inducers.

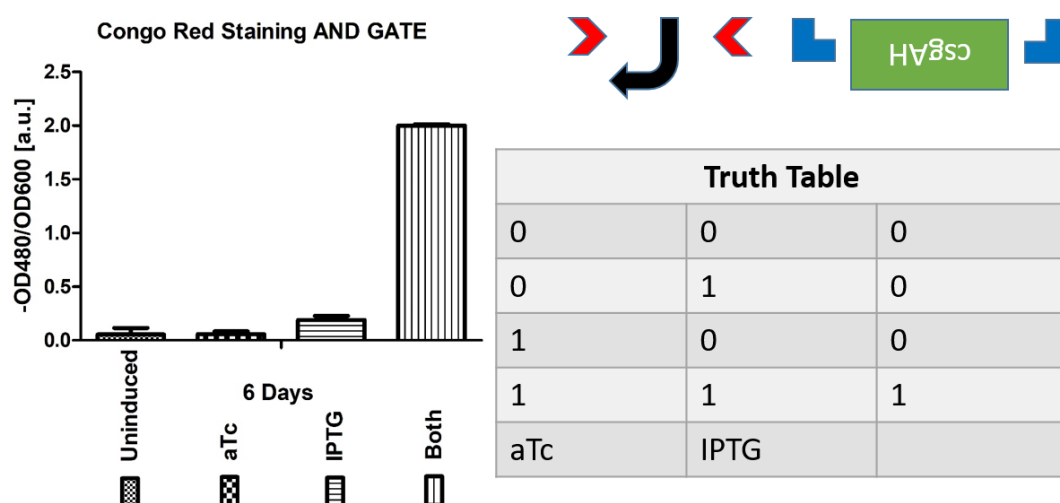


Figure 5. 8: Congo red assay for AND GATE.

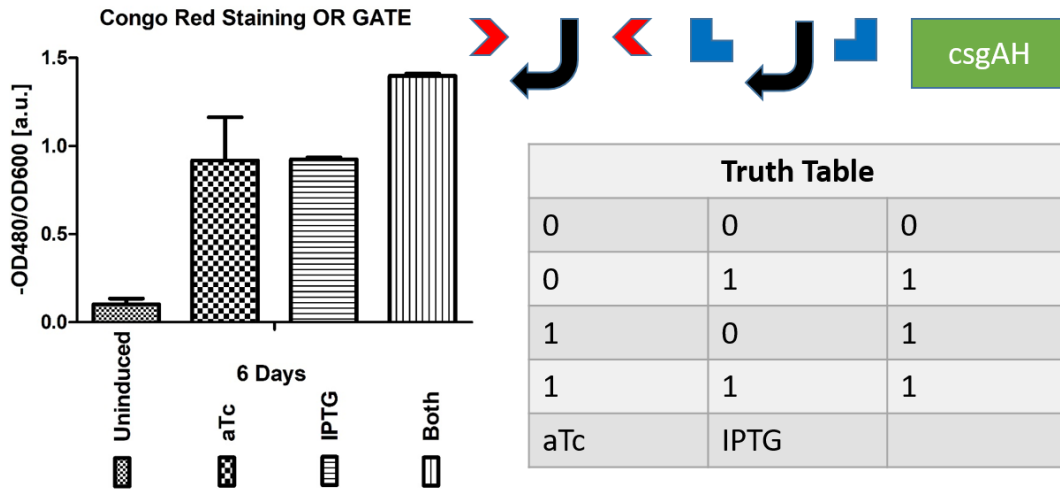


Figure 5. 9: Congo red assay for OR GATE.

5.3.3. Scanning Electron Microscopy Imaging

For the visualization of curli fibers, cells were incubated for 6 days on minimal medium with appropriate inducers and used for scanning electron microscopy imaging.

5.3.3.1. AND GATE

4 different samples (uninduced, aTc-induced, IPTG-induced and both-induced) were analyzed for For the AND GATE. As could be expected, no fibers were observed on uninduced cells (Figure 5.10). We mainly observed cells without curli fibers and some curli leakages on only-aTc and only-IPTG induced cells (Figure 5.11-12). However, cell clusters having dense curli fibers were present following induction with both aTc and IPTG (Figure 5.13).

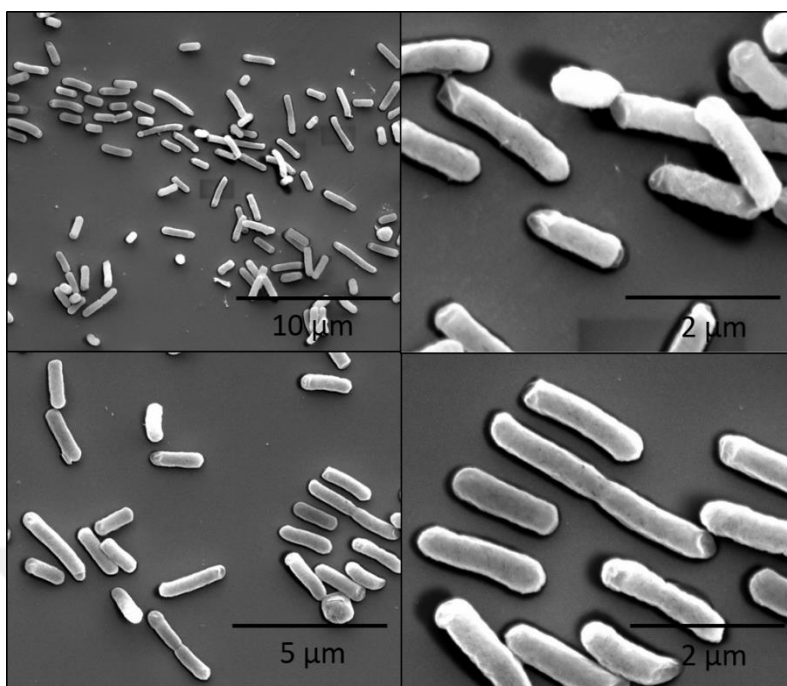


Figure 5.10: Scanning electron microscopy images of uninduced AND GATE cells.

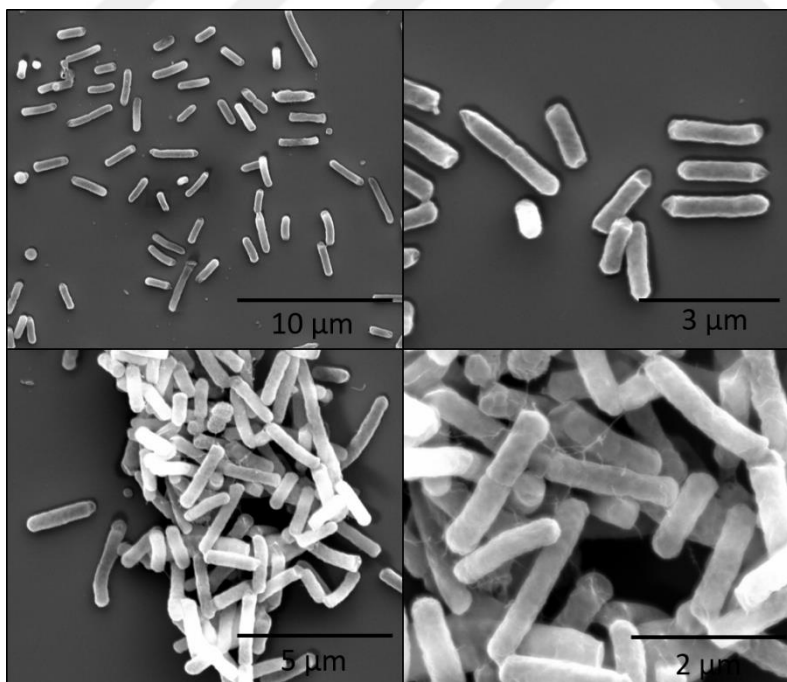


Figure 5.11: Scanning electron microscopy images of aTc induced AND GATE cells.

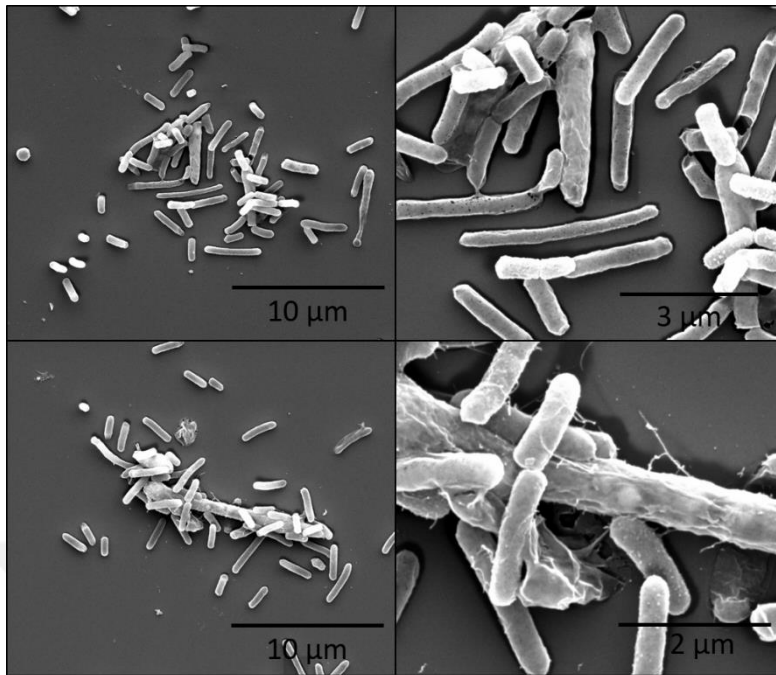


Figure 5.12: Scanning electron microscopy images of IPTG induced AND GATE cells.

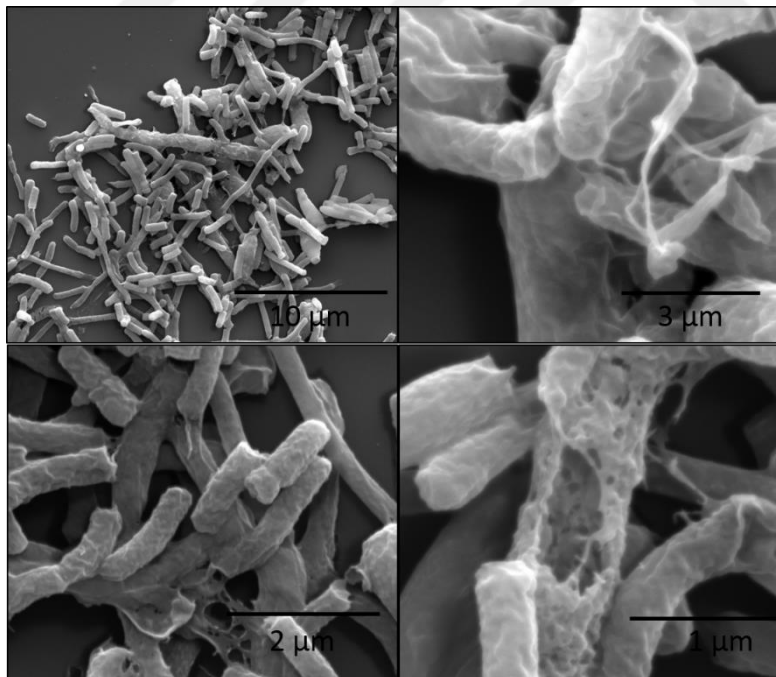


Figure 5.13: Scanning electron microscopy images of aTc/IPTG induced AND GATE cells.

5.3.3.1. OR GATE

4 different samples (uninduced, aTc-induced, IPTG-induced and both-induced) were similarly prepared for the OR GATE. Little to no curli formation was observed on uninduced cells (Figure 5.14). We mainly observed cell clusters with dense curli fibers on only-aTc, only-IPTG and both aTc and IPTG induced cells (Figure 5.15-17).

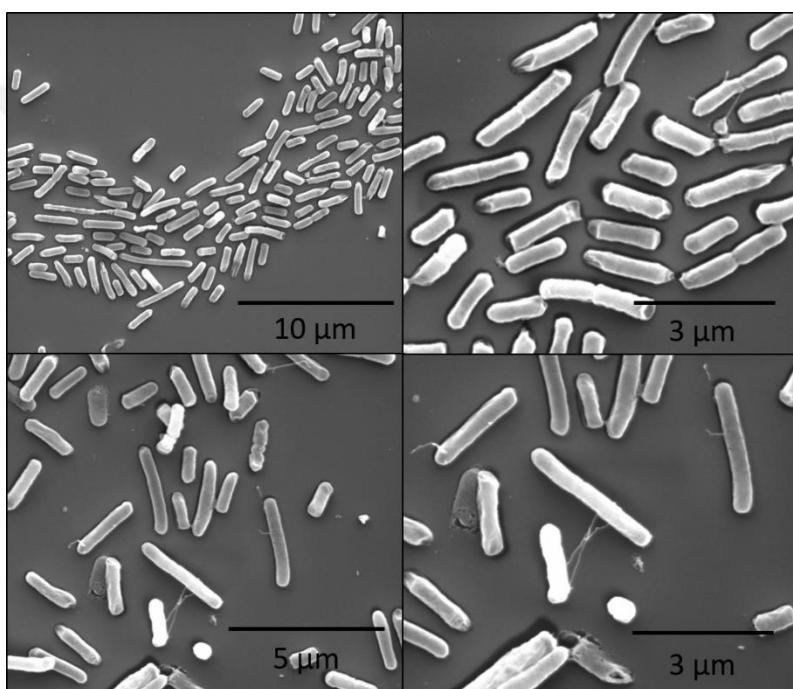


Figure 5.14: Scanning electron microscopy images of uninduced ORGATE cells.

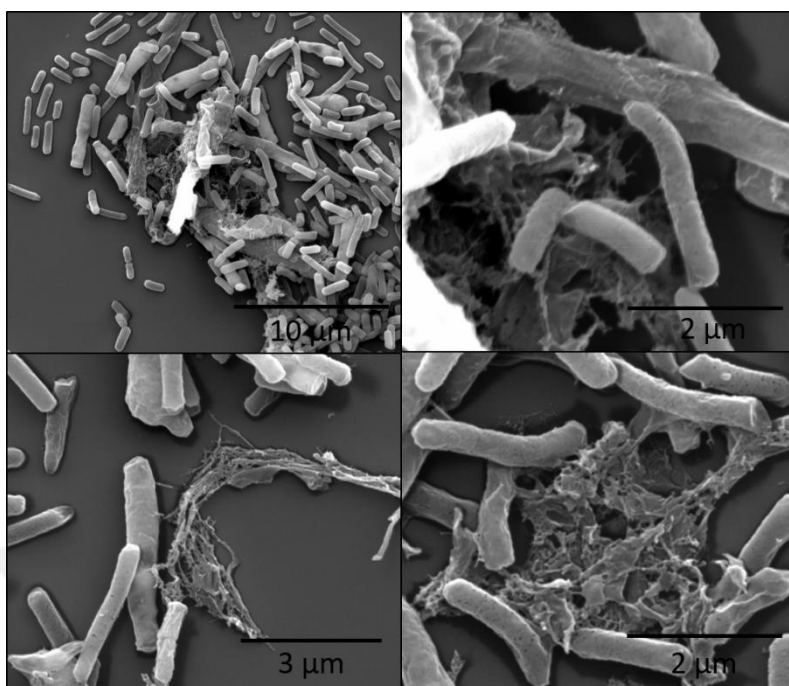


Figure 5.15: Scanning electron microscopy images of uninduced OR GATE cells.

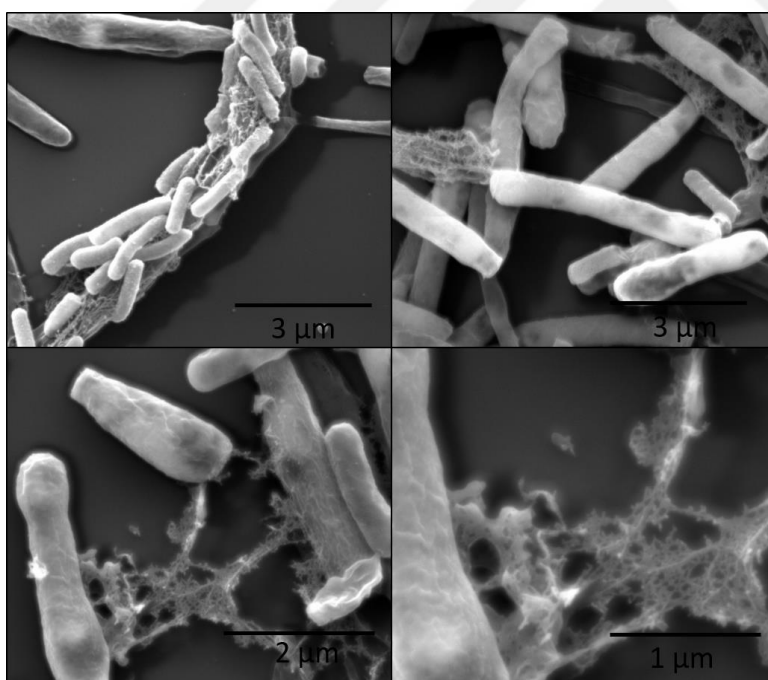


Figure 5.16: Scanning electron microscopy images of IPTG induced ORGATE cells.

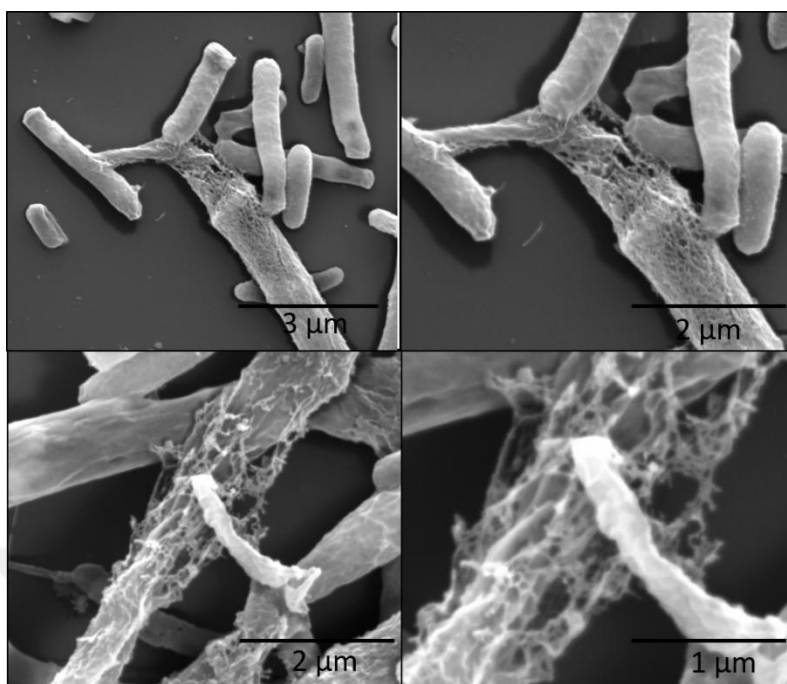


Figure 5.17: Scanning electron microscopy images of aTc/IPTG induced ORGATE cells.

5.4. Discussion

In this chapter of the thesis, we have shown that functional curli fibers by living cells can be controlled by genetic logic gates. Genetic circuits designed with two-input Boolean logic functions were shown in many studies [283, 285-287]. The use of genetic logic gates in engineered biofilms will be useful for the patterning of biofilm surface for novel applications. We characterized the AND GATE and OR GATE logic gates that control the expression of a Histaq-modified version of CsgA protein. This systems can be used as a starting point to build complex circuits that controls the patterning of biofilm.

In the congo red staining part, inducer molecules were added at the beginning of the experiment. We wanted the recombinase enzymes to be expressed early to invert the associated gene regions. Recombinase enzymes invert %90 percent of

the DNA in 16 hours *in vitro* [288]. Therefore; cells can start producing curli protein in the second day of incubation After 3 days of incubation, media of cells were refreshed for better biofilm formation. Congo red staining results were taken at the 6th day of incubation (Figure 5.8-9). AND GATE and OR GATE circuits worked efficiently in the presence of inducer molecules when compared with truth tables. We used tetO promoter and lacO promoter for the expression of recombinase enzymes because our host cell contains tetR and lacI inhibitor genes in its genome. Therefore; leakages from the promoters in plasmids occur at a minimum level, ensuring that gene inversion doesn't happen in uninduced cells. TP901 recombinase is originally controlled by the pBAD promoter, which is induced by arabinose sugar. Arabinose sugar can be used by the cells as a carbon source when incubated in minimal medium, and this might cause the inefficient induction of TP901 recombinase enzyme. Therefore; we replaced the pBAD promoter with lacO promoter, which is repressed by lacI.

SEM imaging of curli fibers required careful sample preparation, because curli fibers can break down or coagulate while preparing the samples. During fixation steps, cells and curli fibers may lose their morphologies [289]. In order to prevent this, we used critical point dryer (CPD) in this chapter. Curli fibers were observed as condensed and spider web-like networks in SEM images (Figures 5.13 and, 5.15-17). The 6-day incubation time may increase the concentration of curli fibers and sample preparation might cause the fibers to collide, as we disrupt the biofilm from the surface in order to prepare the samples for imaging. We also observed higher leakages in the OR GATE uninduced cells (Figure 5.14) compared to AND GATE uninduced cells (Figure 5.10). Expression of a single recombinase enzyme

is sufficient to produce leakages in the OR GATE, as this logic gate has two promoters (Figure 5.2) while the AND GATE has one promoter (Figure 5.1). Inversion of one of the promoter is therefore enough to facilitate curli expression, making the OR GATE circuit leakier than the AND GATE.

5.5. Conclusion

We successfully designed two recombinase based logic gate circuits that control the expression of Histaq modified version of CsgA. AND Gate expresses the CsgA-Histaq in the presence of both inducer molecules and OR Gate expresses the CsgA-Histaq in the presence of either aTc or IPTG inducer.

5.6. Experimental Section

5.6.1. Cloning of AND GATE and OR GATE

All the procedures (plasmid purification, PCR, agarose gel electrophoresis, gel extraction, endonuclease digestion, ligation, Gibson assembly, competent cell preparation and transformation) were performed as indicated in chapter 2.

To produce the AND GATE logic gate circuit (Figure 5.1), *E. coli* codon-optimized inverted csgA-Histaq and inverted proD promoter sequences were artificially synthesized as a gene fragment with homologous regions to pZa-AmpR backbone (Genewiz, USA). Then this part was ligated into pZa-AmpR using Gibson assembly method [290]. To produce OR GATE logic gate circuit (Figure 5.2), inverted csgA-Histaq were replaced with PCR amplified inverted proD sequence, and a PCR amplified csgA-Histaq was cloned into downstream of

inverted proD using Gibson assembly method. To produce Dual recombinase controller vector with pLacO promoter in the upstream of TP901 recombinase gene, pLacO promoter were amplified from pZs2-123 vector (Addgene plasmid# 26598) [284].

Ligation products (Figures C.12-C14) were transformed into chemically competent *E. coli* MG1655 *PRO ΔcsgBA ompR234* host strain by standard transformation procedure as in chapter 2. Colonies were selected on ampicillin or chloramphenicol LB agar plates. Gene inserts were verified with DNA sequencing. Sequencing results are provided on appendix D.

5.6.2. Congo Red Staining

20 µl of overnight cultures of bacteria were inoculated into 2mL M63 medium minimal medium supplemented with 1 mM MgSO₄ and 0.2 % glucose and grown at 30 °C in 24 well plates without shaking for 6 days. Curli fiber production was induced with appropriate inducer (200ng/ml aTc or 0.25mM IPTG) under static or dynamic culture condition, and 1 ml of bacterial culture was subsequently centrifuged down in an Eppendorf tube. M63 medium was replaced with 1xPBS. Then, Congo red solution was added in final concentration of 25 µg/ml. The samples were incubated for 2 hours, centrifuged, and had their absorbance measured at 480nm.

5.6.3. Scanning Electron Microscopy

20 µl of overnight cultures of bacteria were inoculated into 2mL M63 medium minimal medium supplemented with 1 mM MgSO₄ and 0.2 % glucose and grown at 30 °C in 24 well plates without shaking for 6 days. Curli fiber production was induced with appropriate inducer (aTc or IPTG) under static or dynamic culture condition. For sample preparation, biofilm was washed with ddH₂O and fixed with 2.5 % glutaraldehyde overnight at +4 °C. Next day, samples were dehydrated by washing twice in each concentration of ethanol in a graded series (%30, %50, %70, %99) for 5 min followed by sputtering 5nm Au/Pd alloy. Crytical point dryer (CPD) was applied to maintain biofilm structure.

CHAPTER 6

Conclusion & Future Prospects

In this thesis, expression control, functionalization and tuning mechanical properties of genetically engineered curli fibers by using synthetic biology approaches was investigated. In the first part, Curli operon was cloned under inducible genetic circuits to control the expression of curli fibers. These circuits allow us to produce curli fibers without using biofilm conditions.

In the second part, we have investigated the possibility to establish conductive biofilm architectures. Therefore we first screened aromatic amino acids for their conductivities within a nanofiber frame. Best performing peptides were genetically fused into the major biofilm protein CsgA to transform the biofilm into a conductive interface. We showed that non-conductive interfaces of *E.coli* can become conductive network with formation of conductive nanofiber engineering.

In the third part, native CsgA, CsgB and their linker modified versions were investigated with atomic force microscopy. The study showed that minor protein CsgB can be used to tune the mechanical properties of the biofilm structures. Genetically tunable biofabrication of self-assembling functional materials will broaden the utility of biofilm engineering for new applications in materials science and nanotechnology.

In the fourth part, two recombinase based logic gate circuits were designed and built to control the expression of Histaq modified version of CsgA. AND Gate and OR Gate were successfully expressed the CsgA-Histaq in the presence of external molecules. These gates open the possibility of designing two component protein system composed of CsgA and CsgAHistaq under the control of logic gates.

Engineered bacteria that serve as ‘living functional materials’ can be obtained through the use of synthetic biology for applications such as wastewater treatment, environmental remediation and fermentation. Engineered biofilms can likewise be adapted to immobilize toxic metals in soil by precipitation[120].

Biofilm engineering could enhance the ability of naturally occurring bacteria to detoxify wastewaters by introducing the resistance- and sequestering-related genes that are required for treating specific waste products.

Another application of engineered biofilms is the microbial fuel cell (MFC) technology, which entails the production of electricity by microbially catalyzed organic waste. Therefore; it is important to engineer the adhesive, physical or

mechanical properties of biofilm nanomaterials to adapt bacterial cells for optimal performance in MFC applications.

Artificial biofilms could also be used as protective layers for biomaterials (or quick-degrading non-biological materials) to increase their effective lifespan.

Engineered biofilm materials could be combined with genetic circuits to increase the stability and survivability of whole cell biosensor platforms in harsh environmental conditions. In addition, genetic circuit outputs can be linked to electronic circuits through biofilms composed of conductive nanofibers.

Engineered biofilms could also be used to process resources such as solid waste, volatiles, and geological materials in space. As previously mentioned, a combination of light-harvesting strategies and biofilm materials could be used to produce oxygen, fuel and food from available carbon dioxide, sunlight and water on Mars or other planets, aiding in future space research efforts [134].

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APPENDIX A

DNA sequences of constructs used in this study.

Table A.1: Gene sequences of the PCR Products used in chapter 3.

Gene Fragment	Sequence
CsgAW	GGTACCATGAACTTTTAAAAGTAGCAGCAATTGCAGCAA TCGTATTCTCCGGTAGCGCTCTGGCAGGTGTTGTTTCCTCAG TACGGCGGCGGCGGTAACCACGGTGGTGGCGGTAATAATA

	GCGGCCCAAATTCTGAGCTGAACATTTACCAGTACGGTGG CGGTAACCTCTGCACTTGCTCTGCAAACCTGATGCCCCTAACT CTGACTTGACTATTACCCAGCATGGCGGCGGTAATGGTGCA GATGTTGGTCAGGGCTCAGATGACAGCTCAATCGATCTGA CCCAACGTGGCTTCGGTAACAGCGCTACTCTTGATCAGTG GAACGGCAAAAATTCTGAAATGACGGTTAAACAGTTCGGT GGTGGCAACGGTGCTGCAGTTGACCAGACTGCATCTAACT CCTCCGTCAACGTGACTCAGGTTGGCTTTGGTAACAACGC GACCGCTCATCAGTACTACTGGTGGCGTAAATGGAAAGAATGG GATGACTGGTAAACGCGT
CsgAY	GGTACCATGAAACTTTTAAAAGTAGCAGCAATTGCAGCAA TCGTATTCTCCGGTAGCGCTCTGGCAGGTGTTGTTTCCTCAG TACGGCGGCGGCGGTAACCACGGTGGTGGCGGTAATAATA GCGGCCCAAATTCTGAGCTGAACATTTACCAGTACGGTGG CGGTAACCTCTGCACTTGCTCTGCAAACCTGATGCCCCTAACT CTGACTTGACTATTACCCAGCATGGCGGCGGTAATGGTGCA GATGTTGGTCAGGGCTCAGATGACAGCTCAATCGATCTGA CCCAACGTGGCTTCGGTAACAGCGCTACTCTTGATCAGTG GAACGGCAAAAATTCTGAAATGACGGTTAAACAGTTCGGT GGTGGCAACGGTGCTGCAGTTGACCAGACTGCATCTAACT CCTCCGTCAACGTGACTCAGGTTGGCTTTGGTAACAACGC GACCGCTCATCAGTACTACTATCGCAAATACAAAGAGTAT GATGACTACTAAACGCGT
CsgAWY	GGTACCATGAAACTTTTAAAAGTAGCAGCAATTGCAGCAA TCGTATTCTCCGGTAGCGCTCTGGCAGGTGTTGTTTCCTCAG TACGGCGGCGGCGGTAACCACGGTGGTGGCGGTAATAATA GCGGCCCAAATTCTGAGCTGAACATTTACCAGTACGGTGG CGGTAACCTCTGCACTTGCTCTGCAAACCTGATGCCCCTAACT CTGACTTGACTATTACCCAGCATGGCGGCGGTAATGGTGCA GATGTTGGTCAGGGCTCAGATGACAGCTCAATCGATCTGA CCCAACGTGGCTTCGGTAACAGCGCTACTCTTGATCAGTG GAACGGCAAAAATTCTGAAATGACGGTTAAACAGTTCGGT GGTGGCAACGGTGCTGCAGTTGACCAGACTGCATCTAACT CCTCCGTCAACGTGACTCAGGTTGGCTTTGGTAACAACGC GACCGCTCATCAGTACTACTATCGCAAATACAAAGAGTAC GATGATTGGTAAACGCGT
CsgAYW	GGTACCATGAAACTTTTAAAAGTAGCAGCAATTGCAGCAA TCGTATTCTCCGGTAGCGCTCTGGCAGGTGTTGTTTCCTCAG TACGGCGGCGGCGGTAACCACGGTGGTGGCGGTAATAATA GCGGCCCAAATTCTGAGCTGAACATTTACCAGTACGGTGG CGGTAACCTCTGCACTTGCTCTGCAAACCTGATGCCCCTAACT CTGACTTGACTATTACCCAGCATGGCGGCGGTAATGGTGCA GATGTTGGTCAGGGCTCAGATGACAGCTCAATCGATCTGA CCCAACGTGGCTTCGGTAACAGCGCTACTCTTGATCAGTG GAACGGCAAAAATTCTGAAATGACGGTTAAACAGTTCGGT GGTGGCAACGGTGCTGCAGTTGACCAGACTGCATCTAACT CCTCCGTCAACGTGACTCAGGTTGGCTTTGGTAACAACGC GACCGCTCATCAGTACTACTATCGCAAATACAAAGAGTGG

	GATGATTATTAAACGCGT
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Table A.2: Sequence of the PCR products in chapter 4.

PCR product that contained <i>csgA</i> gene fragment (in red) for pZa-CmR-aTc-ribo vector	GGTACCATGAAACTTTTAAAAGTAGCAGCAATTGCA GCAATCGTATTCTCCGGTAGCGCTCTGGCAGGTGTT GTTCTCAGTACGGCGGCGGCGGTAACCACGGTGGT GGCGGTAATAATAGCGGCCCAAATTCTGAGCTGAAC ATTTACCAGTACGGTGGCGGTAACCTCTGCACTTGCT CTGCAAACCTGATGCCCCTAACTCTGACTTGACTATT ACCCAGCATGGCGGCGGTAATGGTGCAGATGTTGGT CAGGGCTCAGATGACAGCTCAATCGATCTGACCC AACGTGGCTTCGGTAACAGCGCTACTCTTGATCAGT GGAACGGCAAAAATTCTGAAATGACGGTTAAACA GTTTCGGTGGTGGCAACGGTGCTGCAGTTGACCAGAC TGCATCTAACTCCTCCGTCAACGTGACTCAGGTT GGCTTTGGTAACAACGCGACCGCTCATCAGTACTAA ACGCGT
PCR product that contained <i>csgB</i> gene fragment (in red) for pZa-CmR-aTc-ribo vector	CCGGGTACATGAAAAACAAATTGTTATTTATGATGT TAACAATACTGGGTGCGCCTGGGATTGCAGCCGCAG CAGGTTATGATTTAGCTAATTCAGAATATAACTTCG CGGTAAATGAATTGAGTAAGTCTTCATTTAATCAGG CAGCCATAATTGGTCAAGCTGGGACTAATAATAGTG CTCAGTTACGGCAGGGAGGCTCAAACTTTTGGCGG TTGTTGCGCAAGAAGGTAGTAGCAACCGGGCAAAG ATTGACCAGACAGGAGATTATAACCTTGCATATATT GATCAGGCGGGCAGTGCCAACGATGCCAGTATTTTCG CAAGGTGCTTATGGTAATACTGCGATGATTATCCA GAAAGGTTCTGGTAATAAAGCAAATATTACACAGTA TGGTACTCAAAAAACGGCAATTGTAGTGCAGAGA CAGTCGCAAATGGCTATTCGCGTGACACAACGTAA ACGCGT
PCR product that contained <i>csgA</i> gene fragment (in red) and linker (in yellow) for pET22b(+) expression vector	GCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCT GCCCAGCCGGCGATGGCCATGGATGAAACTTTTAAA AGTAGCAGCAATTGCAGCAATCGTATTCTCCGGTAG CGCTCTGGCAGGTGTTGTTCTCAGTACGGCGGCGG CGGTAACCACGGTGGTGGCGGTAATAATAGCGGCC AAATTCTGAGCTGAACATTTACCAGTACGGTGGCGG TAACTCTGCACTTGCTCTGCAAACCTGATGCCCCTAA CTCTGACTTGACTATTACCCAGCATGGCGGCGGTAA TGGTGCAGATGTTGGTCAGGGCTCAGATGACAGCTC AATCGATCTGACCCAACGTGGCTTCGGTAACAGCGC TACTCTTGATCAGTGGAACGGCAAAAATTCTGAAAT GACGGTTAAACAGTTCGGTGGTGGCAACGGTGCTGC AGTTGACCAGACTGCATCTAACTCCTCCGTCAACGT

	GACTCAGGTTGGCTTTGGTAACAACGCGACCGCTCA TCAGTACGGTACCGGCGGTAGCGGTGGCGGTAGCG GAGGCGGTAGCGGCGAAAACCTGTATTTTCAGGGCG GCGGTAGCGGTGGCGGTAGCGGAGGCGGTAGCGGC CTCGAGCACCACCACCACCACCTGAGATCCGGCT GCTAACAAAGCCCCGA
PCR product that contained <i>csgB</i> gene fragment (in red) and linker (in yellow) for pET22b(+) expression vector	GCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATGA AAAACAAATTGTTATTTATGATGTTAACAATACTGG GTGCGCCTGGGATTGCAGCCGCAGCAGGTTATGATT TAGCTAATTCAGAATATAACTTCGCGGTAAATGAAT TGAGTAAGTCTTCATTTAATCAGGCAGCCATAATTG GTCAAGCTGGGACTAATAATAGTGCTCAGTTACGGC AGGGAGGCTCAAAACTTTTGGCGGTTGTTGCGCAAG AAGGTAGTAGCAACCGGGCAAAGATTGACCAGACA GGAGATTATAACCTTGCATATATTGATCAGGCGGGC AGTGCCAACGATGCCAGTATTTTCGCAAGGTGCTTAT GGTAATACTGCGATGATTATCCAGAAAGGTTCTGGT AATAAAGCAAATATTACACAGTATGGTACTCAAAAA ACGGCAATTGTAGTGCAGAGACAGTCGCAAATGGCT ATTCGCGTGACACAACGTGGTACCGGCGGTAGCGGT GGCGGTAGCGGAG
PCR product that contained CmR gene fragment(in red), Cat Promoter(in yellow) and overlaps with pET22b(+) vector (lower-case letters)	atgagtaaacttgggtctgacagttaGATATCAAATTA CGCCCCGCC CTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGC ATTCTGCCGACATGGAAGCCATCACAGACGGCATGA TGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCG CCTTGCGTATAATATTTGCCCATGGTGAAAACGGGG GCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCA AAACTGGTGAAACTCACCCAGGGATTGGCTGAGAC GAAAAACATATTCTCAATAAAACCTTTAGGGGAAATA GGCCAGGTTTTACCGTAACACGCCACATCTTGCGA ATATATGTGTAGAACTGCCGGAAATCGTCGTGGTA TTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTC ATGGAAAACGGTGTAACAAGGGTGAACACTATCCC ATATCACCAGCTCACCGTCTTTCATTGCCATACGGA ATTCGGATGAGCATTATCAGGCGGGCAAGAATGT GAATAAAGGCCGGATAAACTTGTGCTTATTTTTCT TTACGGTCTTTAAAAAGGCCGTAATATCCAGCTGAA CGGTCTGGTTATAGGTACATTGAGCAACTGACTGAA ATGCCTCAAAATGTTCTTTACGATGCCATTGGGATA TATCAACGGTGGTATATCCAGTGATTTTTTTCTCCAT TTAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCA AAAAATACGCCCGGTAGTGATCTTATTTTCATTATGG TGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCT CATTTTCGCCAGATATCGACGTCactcttccttttcaatattattga
PCR product that contained pET22b(+) fragment (in red)	CTCATTTTCGCCAGATATCGACGTCactcttccttttcaatattatt gaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaat aaacaaataggggttccgcgcacatttccccgaaaagtccacctgaaattgtaaacg ttaatattttgttaaattcgcgttaaattttgttaaatacagctcatttttaaccaataggcc

and overlaps with CmR gene fragment (capital letters)	gaaatcggcaaaatccctataaatcaaaagaatagaccgagatagggttgagtgtgt tccagtttggaaacagagtcactattaaagAACgtggactccaacgtcaaagggcga aaaaccgtctatcagggcgatggccactacgtgaaccatcacctaatacagttttt gggtcgaggtgccgtaaagcactaaatcgaaccctaaagggagccccgatttaga gcttgacggggaaagccggcgaacgtggcgagaaaggaagggaagaaagcgaaa ggagcgggcgctagggcgctggcaagtgtagcgggtcacgctgcgcgtaaccacca caccgcgcgcttaatgcgcgctacagggcgctcccatcgccaatccgatat agttcctccttcagcaaaaaaccctcaagaccggttagaggcccaaggggttatg ctagtattgctcagcgggtggcagcagccaactcagcttccttcgggctttgtagcag ccggatctcagtggtggtggtggtggtgctcagtgccgccaagctgtgcacgg agctcgaattcggatccgaattaattccgatatccatggccatcgccggctgggcagc gaggagcagcagaccagcagcagcggctggcagcaggtatttcatatgtatatctct tctaaagttaaacaaaattatttctagagggaattgttatccgctcacaaattcccata gtgagtcgtattaatttcggggatcgagatctcgtcctcagccggacgcacgtg gccggcatcaccggcgccacaggtgcggtgctggcgctatatcgccgacatcacc gatggggaagatcgggctcgccacttcgggctcatgagcgttgttcggcggtgggta tggtggcaggccccgtggccgggggactgttgggcgccatctccttgatgcaccatt ccttgccggcggtgtcaacggcctcaacctactactgggctgcttctaagcag gagtcgcataaggagagcgtcgagatcccggacaccatgaatggcgcaaaacct ttcgcggtatggcatgatagcgccggaagagagtcattcaggggtggtgaatgtgaa accagtaacgttatacgtatgcagagatgccggtgtctctatcagaccgtttccg cgtggtgaaccaggccagccacgtttctgcgaaaacgcgggaaaaagtgaagcgg cgatggcggagctgaattacattcccaaccgcgtggcacaacaactggcgggcaaa cagtcgttgctgattggcgttgccacctcagtcctggccctgcacgcgcctgcgaaat gtcgcggcgattaaatctcgcgcgatcaactgggtgccagcgtggtggtgctgatg gtagaacgaagcggcgtcgaagcctgtaaagcggcggtgcacaatcttctcgcgcaa cgctcagtgggctgattacataactatccgctggatgaccaggatgcattgtgtgga agctgcctgcactaatgttcggcgttatttctgatgtctctgaccagacaccatcaac agtattattttctccatgaagacggtacgcgactgggcgtggagcatctggctgcattg ggtcaccagcaaatcgcgctgttagcgggccattaagtctgtctcggcgcgctgc gtctggctggctggcataaatatctcactcgcaatcaaattcagccgatagcggaacg ggaaggcgactggagtccatgtccggtttcaacaaacctgcaaatgctgaatgag ggcatcgttccactgcgatgctggttccaacgatcagatggcgctggcgcaatgc gcgccattaccgagtcgggctgcgcgttggtgcggatatctcggtagtgggatacga cgataccgaagacagctcatgttatatcccgcgttaaccaccatcaaacaggatttc gcctgctggggcaaacagcgtggaccgcttgctgcaactctctcagggccaggcg gtgaagggaatcagctgttgccgtctcactggtgaaaagaaaaaccacctggcg cccaatacgcaaacgcctctcccgcgcttgccgattcattaatgcagctggcac gacaggtttcccactggaaagcgggcagtgagcgcaacgaattaatgaagttag ctcactcattaggcaccgggatctcagccgatgcccttgagagccttcaaccagtc gctcctccgggtggcgcggggcatgactatcgtcgccgacttatgactgtcttctta tcatgcaactcgtaggacaggtgccggcagcgctctgggtcatttcggcgaggacc gcttctgctggagcgcgacgatgatcgccctgtcgttgcggtattcggaaatttcac gccctcgctcaagccttcgctactggtcccgccacaaacgtttcggcgagaagcag gccattatcgccggcatggcgccccacgggtgcgcatgatcgtgctcctgtcgtga ggaccggctaggctggcggggtgcttactggttagcagaatgaatcaccgatac gcgagcgaaactgaagcgactgctgctgcaaaacgtctgcgacctgagcaacaaca tgaatggtcttcggttccgtgttcgtaaaagtctggaaacgcgggaagtgcgcctgc
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	accattatgttccggatctgcatcgaggatgctgctggctaccctgtggaacacctac atctgtattaacgaagcgtggcattgaccctgagtgattttcttgggtcccgccgcatc cataccgccagttgtttaccctcacaacgttccagtaaccgggcatgttcatcatcagta accggtatcgtgagcatcctctctcgtttcatcggtatcattacccccatgaacagaaatc ccccttacacggaggcatcagtgaccaaacaggaaaaaacggcccttaacatggccc gctttatcagaagccagacattaacgcttctggagaaactcaacgagctggacgcgga tgaacaggcagacatctgtgaatcgcttcacgaccacgctgatgagctttaccgcagct gcctcgcgctttcgggtgatgacgggtgaaaacctctgacacatgcagctcccgagaga cggtcacagcttgctgtaagcggatgccgggagcagacaagcccgtagggcgcg tcagcgggtgttggcgggtgtcggggcgagccatgaccagtcacgtagcgatag cggagtgtatactggcttaactatgcggcatcagagcagattgtactgagagtgcacca tatatgcgggtgtgaaataccgcacagatgcgtaaggagaaaaataccgcacagggcgt cttcgcttctcgtcactgactcgtcgtcggctggtcgttggctgcggcgagcggta tcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcagga aagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgt tgctggcggttttccataggctccgccccctgacgagcatcacaataatcgacgctca agtcagagggtggcgaaacccgacaggactataaagataaccaggcggttccccctgga agtcctctcgtgcgtctcctgttccgaccctgccgcttaccggatacctgtccgcctt ctcccttcgggaagcgtggcgcttctcatagctcacgctgtaggtatctcagttcggtg taggtcgttcgtccaagctgggctgtgtgcacgaacccccgttcagcccagcgct gcgccttatccggtaactatcgtcttgagtccaaccggtaagacacgacttatcgcca ctggcagcagccactggtaacaggattagcagagcagaggtatgtaggcgggtgtaca gagttctgaagtgggtggcctaactacggctacactagaaggacagtatgttgatctgc gctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaca aaccaccgctggtagcgggtggtttttgttgcaagcagcagattacgcgcagaaaaa aaggatctcaagaagatccttgatctttctacggggctgacgctcagtggaacgaaa actcacgttaagggattttggtcatgagattatcaaaaaggatcttcacctagatccttta aattaaaaatgaagttttaaatcaatctaagtatatagagtaaacttggtctgacagtta GATATCAAATTACGCCCCGCCCTGC
--	---

Table A.3: Sequence of the logic gates in chapter 5.

AND LOGIC GATE	ctgataattgccaacacaattaacatctcaatcaaggtaaagtcttttcgttttaaa gttaaacaaaattattttagagggaaaccgttggtgtcctgaatatattatac gagccttatgcatgcccgtaaagtatccagcaaccactcatagacctagggca gcagatagggacgacgtgggttagctgtgaaaggagtttttagttacctaatt gaaataaacgaaataaaaaactcgcaattaagcgagttggagtcgtggttgtctg gtcaaccaccgcggctcagtggtgtacgggtacaaaccccgacggtagcgaga
----------------	--

	gcgttcaccgacaaacaacagataaaacgaaaggccagctcttcgactgagc cttcgttttatttgatgcctggtagtggtgggtgggtgggtggtactgatgagcg gtcgcgttggtaccaaagccaacctgagtcacgttgacggaggagtagatgca gtctggtaactgcagcaccgttgccaccaccgaactgtttaaccgtcatttcag aattttgccgttcactgatcaagagtagcgtgttaccgaagccacgttgggtc agatcgattgagctgtcatctgagccctgaccaacatctgcaccattaccgccgc catgctgggtaatagtcaggtacgggcatcagtttgagagcaagtgcagagttaccgccaccgtactggtaaattgtcagctcagaatttgggccgctatt attaccgccaccaccgtgggtaccgccgccgctactgaggaacaacacctg ccagagcgtaccggagaatacattgctgcaattgctgctacttttaaagtctc atggatcccttctgtgtgacttaaggcccgatgatcctgacgacggagacc gccgtcgtcgacaagccggccga
OR LOGIC GATE	ctgataattgccaacacaattaacatctcaatcaaggtaaattgcttttcgttttaa gttaaacaaaattattttagagggaaaccgttggtgtcctgaatatattatac gagccttatgcatgcccgtaaagtattccagcaaccactcatagacctagggca gcagatagggacgacgtgggttagctgtgaaaggagtttttagttacctaatt gaaataaacgaaataaaaaactcgcaattaagcgagttggagtcgtggtttgtctg gtcaaccaccgcggtctcagtggtgtacggtacaaaccccgacggtacaaagt taaacaaaattattttagagggaaaccgttggtgtcctgaatatattatacga gccttatgcatgcccgtaaagtattccagcaaccactcatagacctagggcagc agatagggacgacgtgggttagctgtgtaaggcccgatgatcctgacgac ggagaccgccgtcgtcgacaagccggccgacccggcttaagtcacacagga aagggatccatgaaacttttaaagttagcagcaattgcagcaatcgatttctccg

	gtagcgctctggcaggtgtgttcctcagtagggcgggcggtaccacggt ggtggcggtataatagcgcccaaattctgagctgaacattaccagtaggt ggcggtaactctgcacttgctctgcaaactgatgccgtaactctgacttgactat taccagcatggcggtgaatggtgcagatgttggtcagggctcagatgaca gctcaatcgatctgaccaacgtggcttcggtaacagcgctactcttgatcagtg gaacggcaaaaattctgaaatgacggttaaacagttcgggtgggcaacgggtg ctgcagttgaccagactgcatctaactcctccgtaacgtgactcaggttggttt ggtaacaacgcgaccgctcatcagtagccaccaccaccaccactaaccag gcatcaataaaacgaaaggctcagtcgaaagactgggccttcgtttatctgtt gtttgtcgggtgaacgctctcgggtacc
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APPENDIX B

List of primers used in this study.

Table B.1: Primers that were used in chapter 2.

csgA	KpnI-csgA-f	GGTACCATGAACTTTTAAAAGTAGCAGCAA TTGC
	MluI-csgA-R	ACGCGTTTAGTACTGATGAGCGGTCGC
csgB	KpnI-csgB-f	CCGGGTACCATGAAAAACAAATTGTTATTTA TGAT
	MluI-csgB-R	GTTACGCGTTTAACGTTGTGTCACGC

csgBA	Forward primer	GAAAGGTACCATGAAAAACAAATTGTTATTT ATGATGTTAACAATACTGGGTGCGC
	Reverse primer	CCACGCGTTTAGTACTGATGAGCGGTCGCG
csgE	Forward primer	TCGGGCCCTGAGGCCTGCAGGGATCTTAGAA TTCATCATGCGCCAAATCG
	Reverse primer	AACCGATTTTTTAATGTACAAAGGAGATGAAA CGTTATTTACGCTGGATTG
csgF	Forward primer	AATAACGTTTCATCTCCTTTGTACATTAAAA ATCGGTTGAGTTATTTTGTAACCCGAAACC
	Reverse primer	CACCGGAATCCTGAAAGCTTGGATCGTCGAC AAGGAGATGCGTGTCAAACATGCAGTAGTTC TACT

Table B.2: Primers that were used in chapter 3.

EK F-W	GGTACCATGAAACTTTTAAAAGT AGCAGCAATTGC	Forward primer for all the PCR reactions
EK R- csgA-W1	TTCTTTCCATTTACGCCACCAGTA CTGATGAGCGGTCGCGTTGTTA	First PCR reaction to amplify csgA gene fragment from pZa- TetO-csgA-CmR and to add conductive domain sequences to 3 prime of gene fragment
EK R- csgA-W2	ACGCGTTTACCAGTCATCCCATTC TTTCCATTTACGCCACCAGTACTG ATG	Second PCR reaction to elongate conductive domain sequences to 3 prime of csgA gene fragment
EK R- csgA-Y1	CTCTTTGTATTTGCGATAGTAGTA CTGATGAGCGGTCGCGTTGTTA	First PCR reaction to amplify csgA gene fragment from pZa- TetO-csgA-CmR and to add conductive

		domain sequences to 3 prime of gene fragment
EK R- csgA-Y2	ACGCGTTTAGTAGTCATCATACTC TTTGTATTTGCGATAGTAGTACTG ATGAGCGG	Second PCR reaction to elongate conductive domain sequences to 3 prime of <i>csgA</i> gene fragment
EK R- csgA-WY1	CTCTTTGTATTTGCGATAGTAGTA CTGATGAGCGGTCGCGTTGTTA	First PCR reaction to amplify <i>csgA</i> gene fragment from pZa-TetO- <i>csgA</i> -CmR and to add conductive domain sequences to 3 prime of gene fragment
EK- R-csgA-WY2	ACGCGTTTAATAATCATCCCACTC TTTGTATTTGCGATAGTAGTACTG ATGAGCGG	Second PCR reaction to elongate conductive domain sequences to 3 prime of <i>csgA</i> gene fragment
EK R- csgA-YW1	CTCTTTGTATTTGCGATAGTAGTA CTGATGAGCGGTCGCGTTGTTA	First PCR reaction to amplify <i>csgA</i> gene fragment from pZa-TetO- <i>csgA</i> -CmR and to add conductive domain sequences to 3 prime of gene fragment
EK R- csgA-YW2	ACGCGTTTACCAATCATCGTACTC TTTGTATTTGCGATAGTAGTACTG ATGAGCGG	Second PCR reaction to elongate conductive domain sequences to 3 prime of <i>csgA</i> gene fragment

Table B.3: Primers used in chapter 4 to build constructs.

Primers	Sequence
native <i>csgA</i> gene amplification forward primer F1	GGTACCATGAACTTTTAAAAGTAGCA GCAATTGC
native <i>csgA</i> gene amplification	ACGCGTTTAGTACTGATGAGCGGTCGC

reverse primer R1	
native <i>csgB</i> gene amplification forward primer F2	CCGGGTACCATGAAAAACAAATTGTTA TTTATGAT
native <i>csgB</i> gene amplification reverse primer R2	GTTACGCGTTTAAACGTTGTGTCACGC-
<i>csgA</i> –modified forward SET1 (codes for CsgAM)- F3	GCTCCTCGCTGCCCAGCCGGCGATGGC CATGGATGAAACTTTTAAAAGTAGCAG CAATTGCAGCAATCGT
<i>csgA</i> –modified reverse SET1 (codes for CsgAM) R3	AAAATACAGGTTTTTCGCCGCTACCGCC TCCGCTACCGCCACCGCTACCGCCGGT ACCGTACTGATGAGCGGTCGCGTTGTT
<i>csgA</i> –modified forward SET2 (codes for CsgAM)- F4	GCTGCCGACCGCTGCTGCTGGTCTGCT GCTCCTCGCTGCCCAG
<i>csgA</i> –modified reverse SET2 (codes for CsgAM) R4	GTGCTCGAGGCCGCTACCGCCTCCGCT ACCGCCACCCTACCGCCGCCCTGAAAA TACAGGTTTTTCGCCGCT
<i>csgB</i> –modified forward SET1 (codes for CsgBM)- F5	GCTCCTCGCTGCCCAGCCGGCGATGGC CATGGATGAAAAACAAATTGTTATTTA TGATGTT
<i>csgB</i> –modified reverse SET1 (codes for CsgBM) R5	CTCCGCTACCGCCACCGCTACCGCCGG TACCACGTTGTGTCACGCGA
<i>csgB</i> –modified forward SET2 (codes for CsgAM)- F6	ATGAGTAACTTGGTCTGACAGTTAGATAT CAAATTACGCCCCGCCCTGC

<i>csgB</i> –modified reverse SET2 (codes for CsgBM) R6	TCAATAATATTGAAAAAGGAAGAGTGACG TCGATATCTGGCGAAAATGAG
Amplification of backbone forward F7	CTCATTTTTCGCCAGATATCGACGTCCTC TTCCTTTTTCAATATTATTGA
Amplification of backbone reverse R7	GCAGGGCGGGGCGTAATTTGATATCTAA CTGTCAGACCAAGTTTACTCAT



Table B.4: Primers used in chapter 5 to build constructs.

Inverted proD	EK INV proD Fwd	TGTACGGTACAAACCCCGACGGTACA AAGTTAAACAAAATTATTTGTAGAGGG AA
	EK INV proD Rev	TCTCCGTCGTCAGGATCATCCGGGCCT TAACACAGCTAACACCACGTCGT
CsgA-Histaq	EK csgA-H Fwd	CCGTTCGTCGACAAGCCGGCCGACCCG GCTTAAGTCACACAGGAAAGGGATCC
	EK csgA-H Rev	AGTGAGCGAGGAAGCGGAATATATCC CCCGGGGTACCGAGAGCGTTCACCG
Dual Recombinase Contoller Backbone	DRC Fwd	GCAGAAAATAAGGAGGAAAAAAAAT GAAACATCATCACCATCACCACCAGG
	DRC Rev	TGCAGGGCGGCCGCCATATGATGCCA AAAAGACAATGAACCCCC
pLlacO	pLlacO F	TATTATTTCTGCAGGGCGGCCGCCAT ATGATGCCAAAAGACAATGAAC
	pLlacO R	GGCCTGGTGGTGATGGTGATGATGTTT CATTTTTTTTCCTCCTTATTTTC

APPENDIX C

Plasmid maps used in this study.

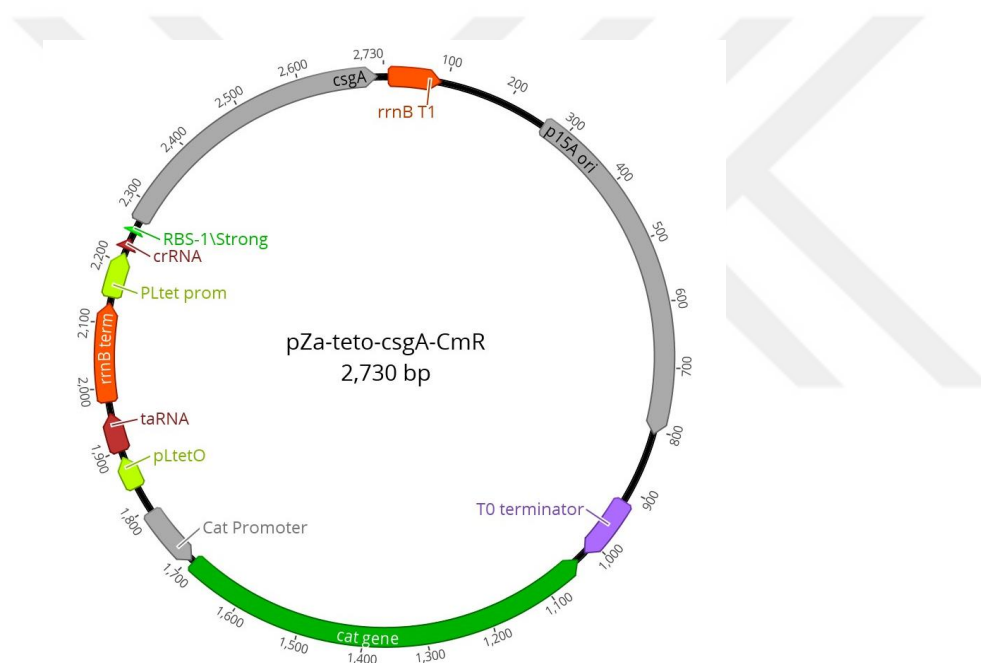


Figure C.1: Schematic representation of pZa vector containing *csgA* gene.

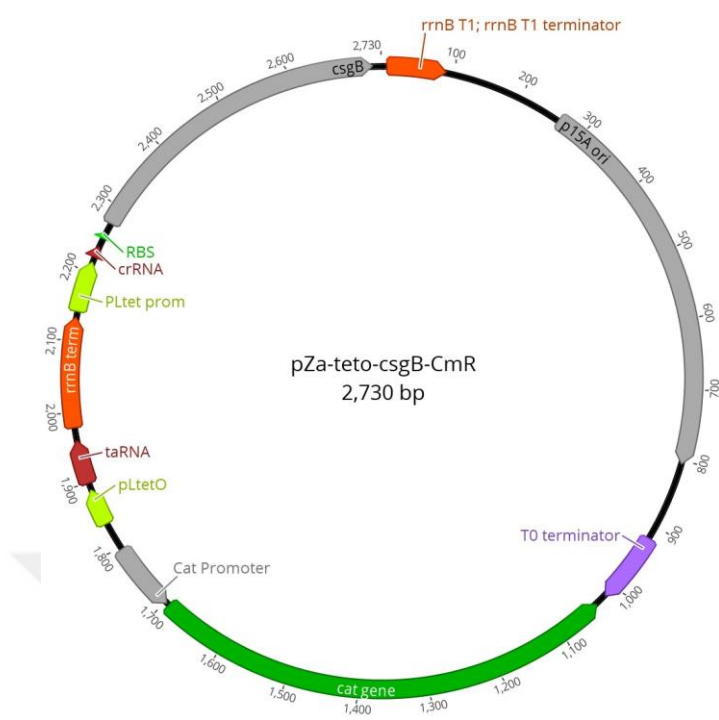


Figure C.2: Schematic representation of pZa vector containing *csgB* gene.

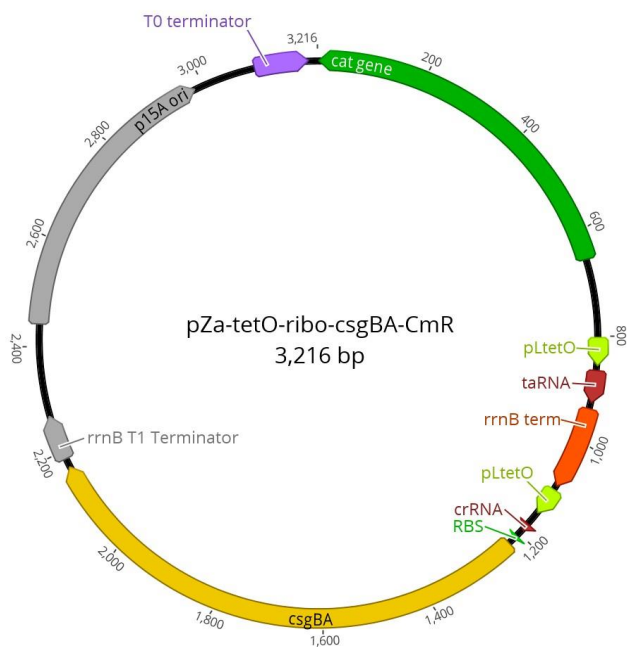
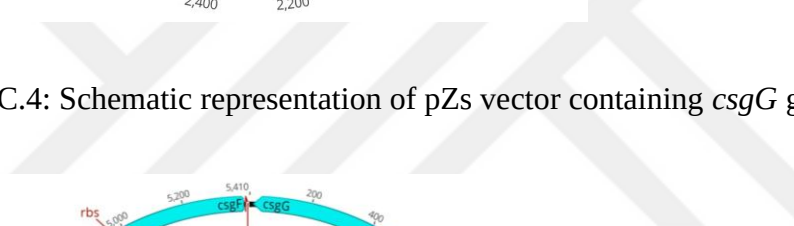
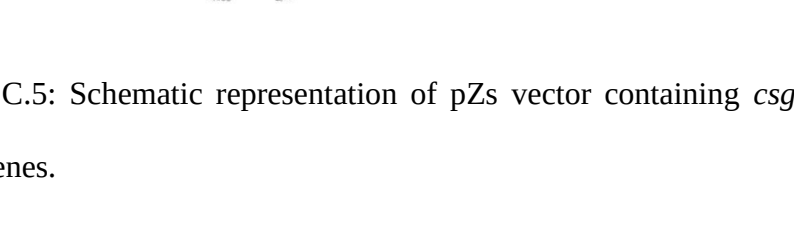


Figure C.3: Schematic representation of pZa vector containing *csgBA* gene.



C.4: Schematic representation of pZs vector containing *csgG* gene



C.5: Schematic representation of pZs vector containing *csgr* genes.

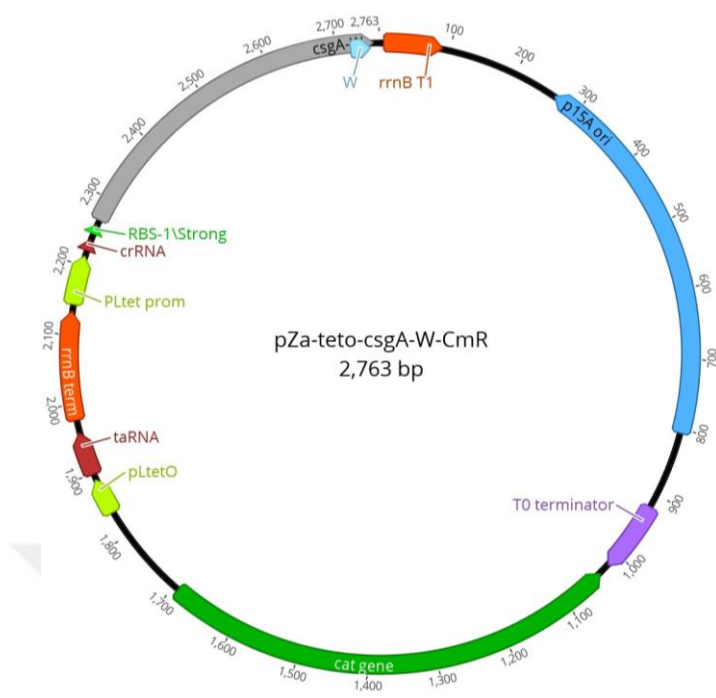


Figure C.6: Schematic representation of the expression vector containing csgAW gene fragment.

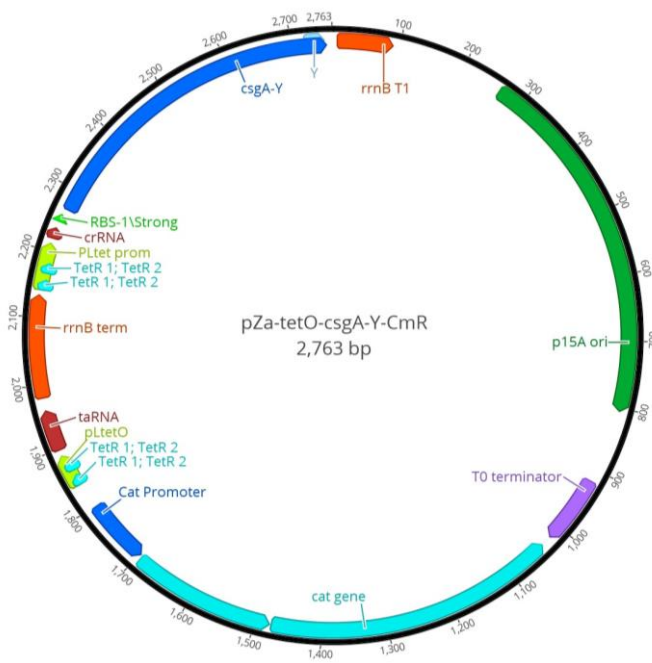


Figure C.7: Schematic representation of the expression vector containing csgAY gene fragment.

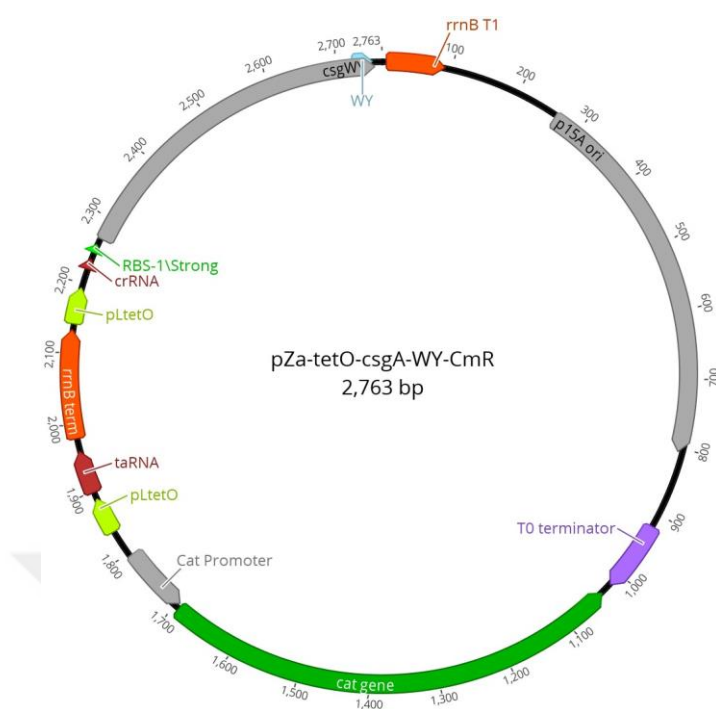


Figure C.8: Schematic representation of the expression vector containing csgAWY gene fragment.

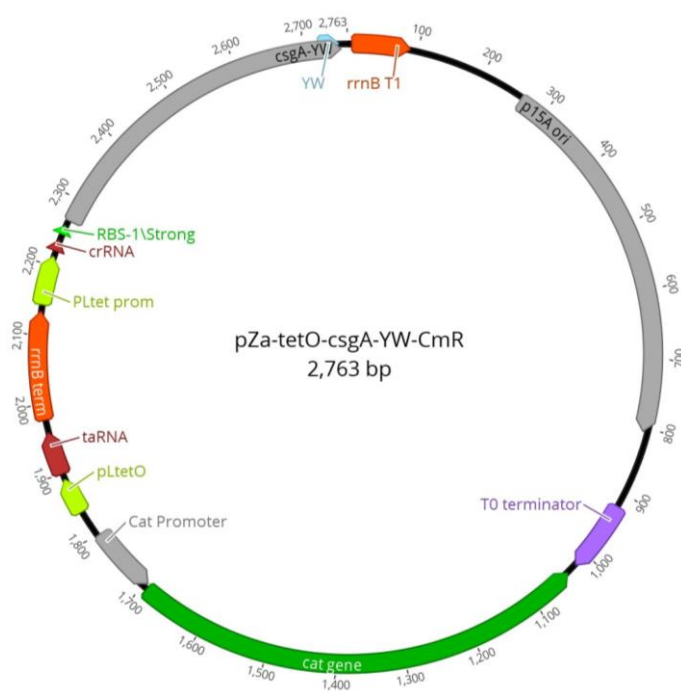


Figure C.9: Schematic representation of the expression vector containing csgAYW gene fragment.

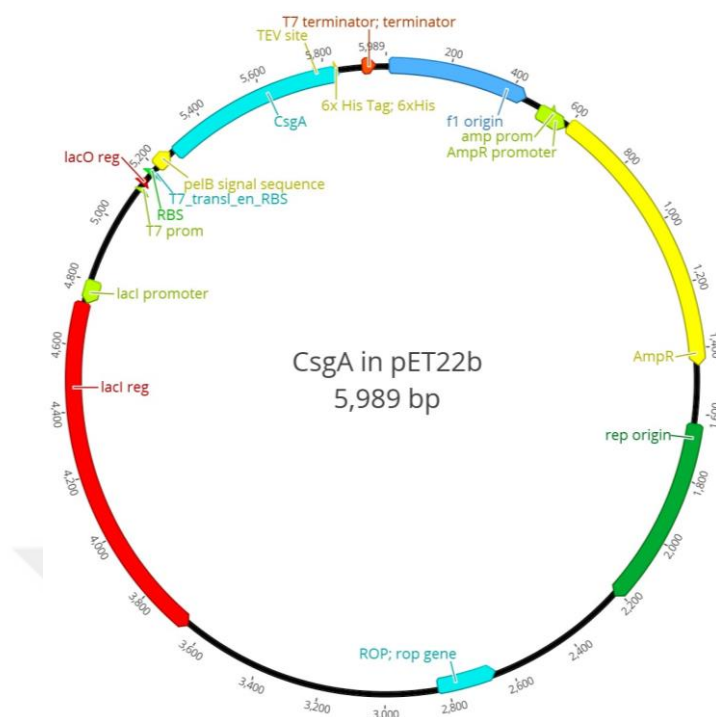


Figure C.10: Schematic representation of plasmid construct that was used to over-express CsgA proteins.

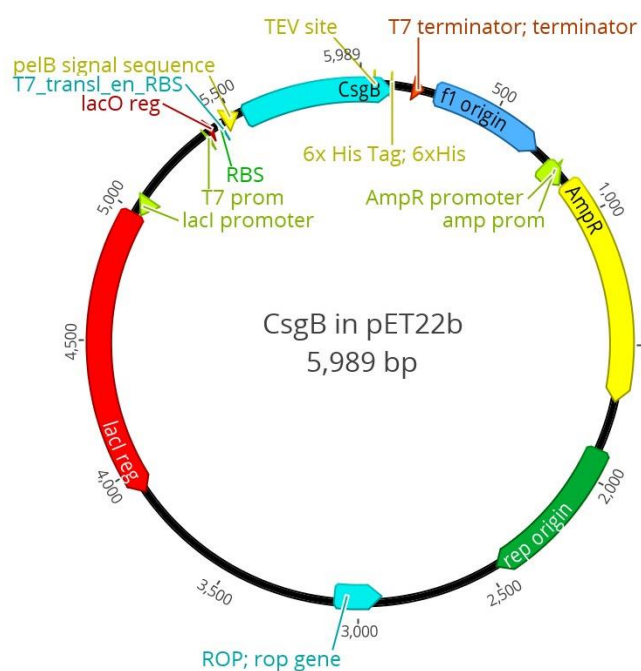


Figure C.11: Schematic representation of plasmid construct that was used to over-express CsgB proteins.

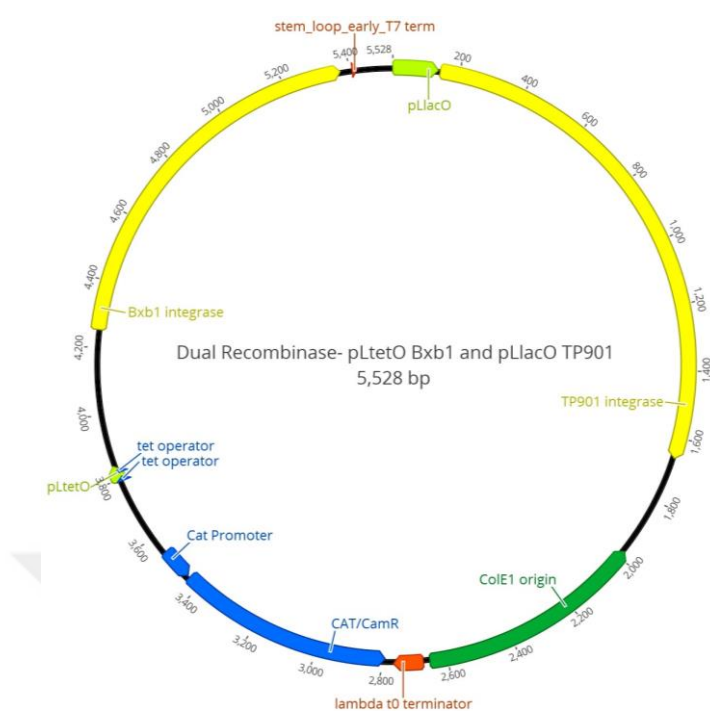


Figure C.12: Schematic representation of Dual Recombinase pLtetO Bxb1/pLlacO TP901 vector.

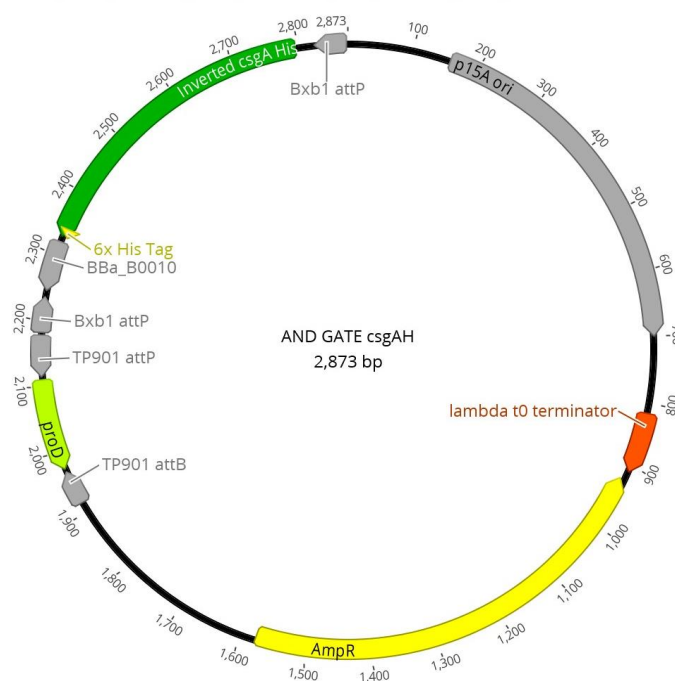


Figure C.13: Schematic representation of AND GATE csgAH.

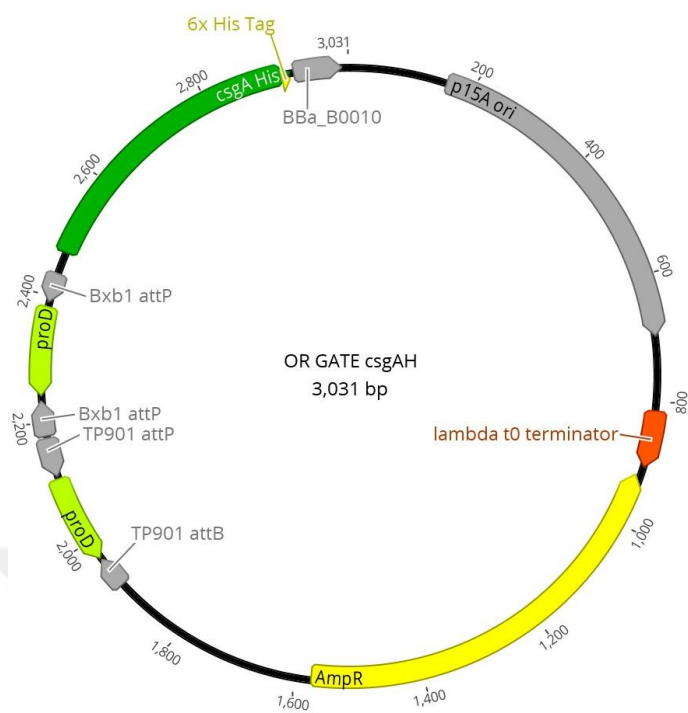


Figure C.14: Schematic representation of OR GATE csgAH.

APPENDIX D

Sanger sequencing results of the plasmids in this thesis.

```

Query 1 ATGAAACTTTTAAAAGTAGCAGCAATTGCAGCAATCGTATTCTCCGGTAGCGCTCTGGCA 60
      |||||||
Sbjct 436 ATGAAACTTTTAAAAGTAGCAGCAATTGCAGCAATCGTATTCTCCGGTAGCGCTCTGGCA 377

Query 61 GGTGTTGTTCTCAGTACGGCGGCGGGTAACCACGGTGGTGGCGGTAATAATAGCGGC 120
      |||||||
Sbjct 376 GGTGTTGTTCTCAGTACGGCGGCGGGTAACCACGGTGGTGGCGGTAATAATAGCGGC 317

Query 121 CCAAATTCTGAGCTGAACATTTACCAGTACGGTGGCGGTAACCTGCACTTGCTCTGCAA 180
      |||||||
Sbjct 316 CCAAATTCTGAGCTGAACATTTACCAGTACGGTGGCGGTAACCTGCACTTGCTCTGCAA 257

Query 181 ACTGATGCCCCGTAACCTGACTTGACTATTACCCAGCATGGCGGCGGTAATGGTGCAGAT 240
      |||||||
Sbjct 256 ACTGATGCCCCGTAACCTGACTTGACTATTACCCAGCATGGCGGCGGTAATGGTGCAGAT 197

Query 241 GTTGGTCAGGGCTCAGATGACAGCTCAATCGATCTGACCCAACGTGGCTTCGGTAACAGC 300
      |||||||
Sbjct 196 GTTGGTCAGGGCTCAGATGACAGCTCAATCGATCTGACCCAACGTGGCTTCGGTAACAGC 137

Query 301 GCTACTCTTGATCAGTGGAAACGGCAAAAATTCTGAAATGACGGTTAAACAGTTCGGTGGT 360
      |||||||
Sbjct 136 GCTACTCTTGATCAGTGGAAACGGCAAAAATTCTGAAATGACGGTTAAACAGTTCGGTGGT 77

Query 361 GGCAACGGTGCTGCAGTTGACCAGACTGCATCTAACTCCTCCGTCAACGTGACTCAG 417
      |||||||
Sbjct 76 GGCAACGGTGCTGCAGTTGACCAGACTGCATCTAACTCCTCCGTCAACGTGACTCAG 20

```

Figure D.1: Sequence alignment of cloned *csqA* gene fragment in pZa vector. Aligned sequences are linked each other. Upper DNA sequence is the reference and lower one is the cloned DNA sequence.

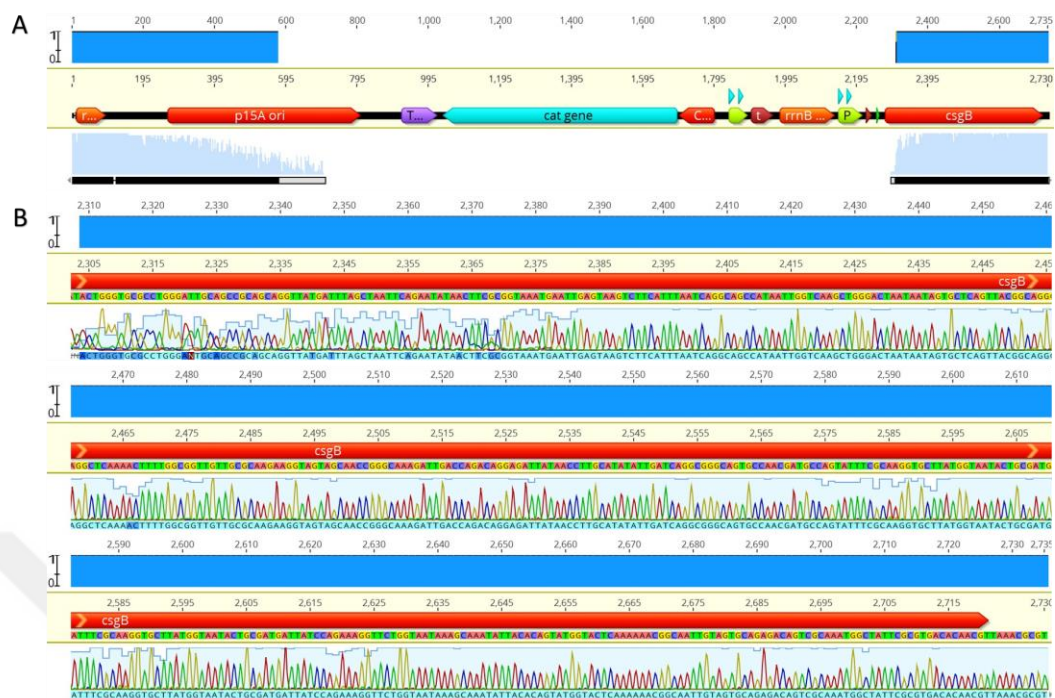


Figure D.2: Sanger DNA sequencing results of *csgB* in pZa vector. A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the blue bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.

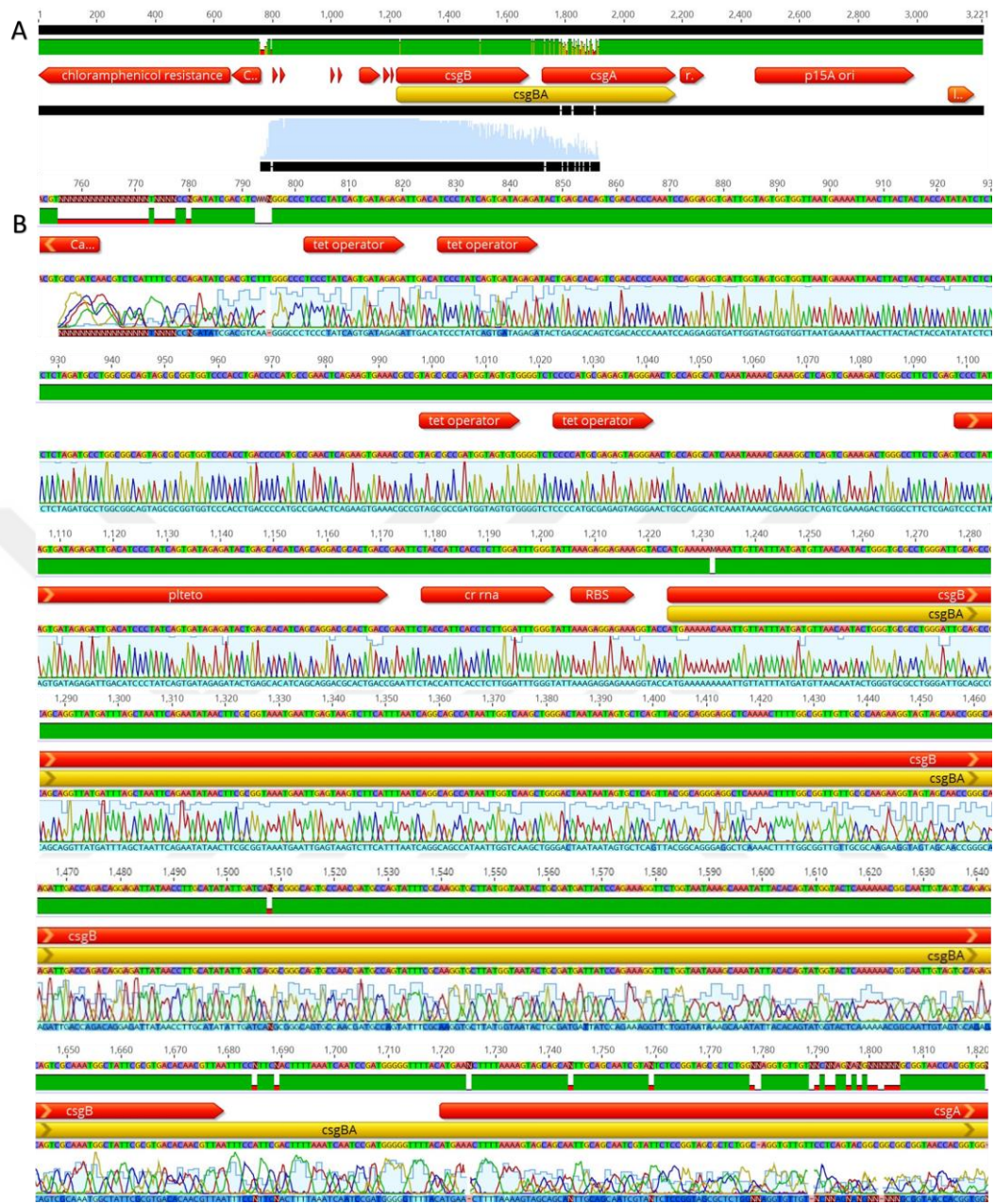


Figure D.3: Sanger DNA sequencing results of *csgBA* in pZa vector. A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the green bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.

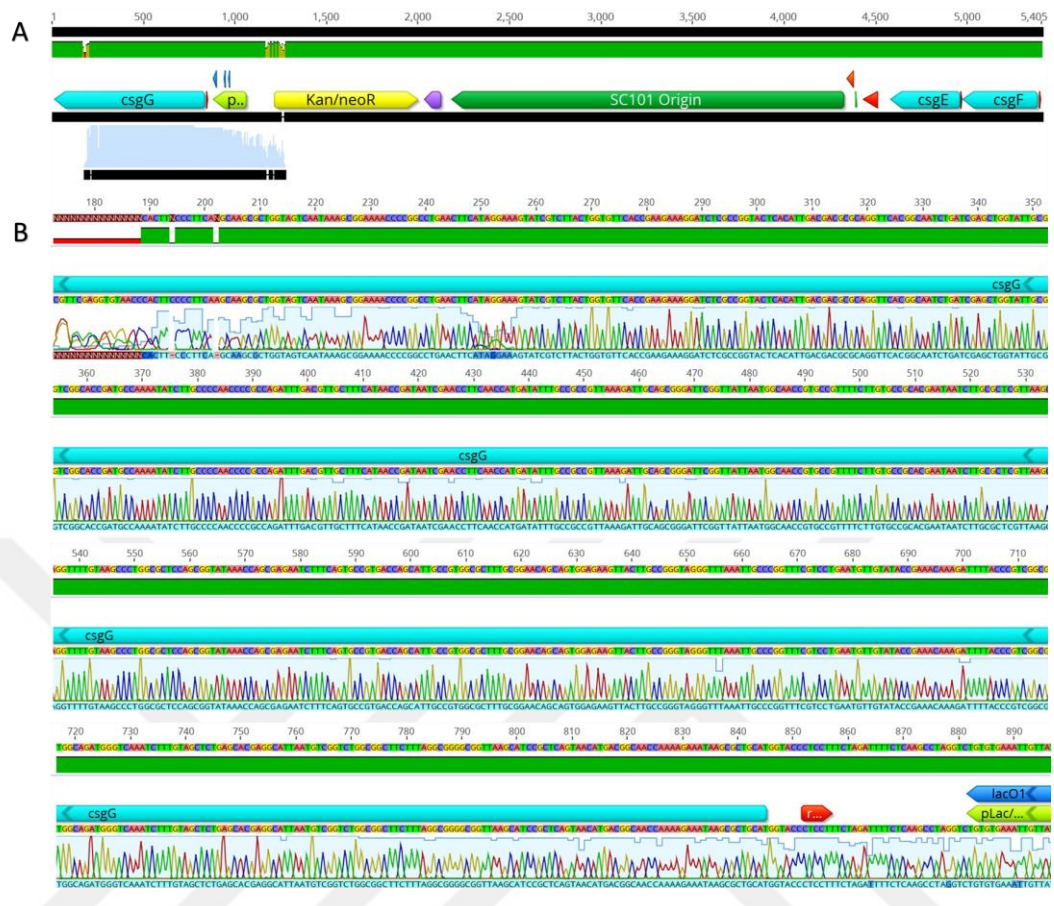


Figure D.4: Sanger DNA sequencing results of *csgG* in pZs vector. A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the green bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.

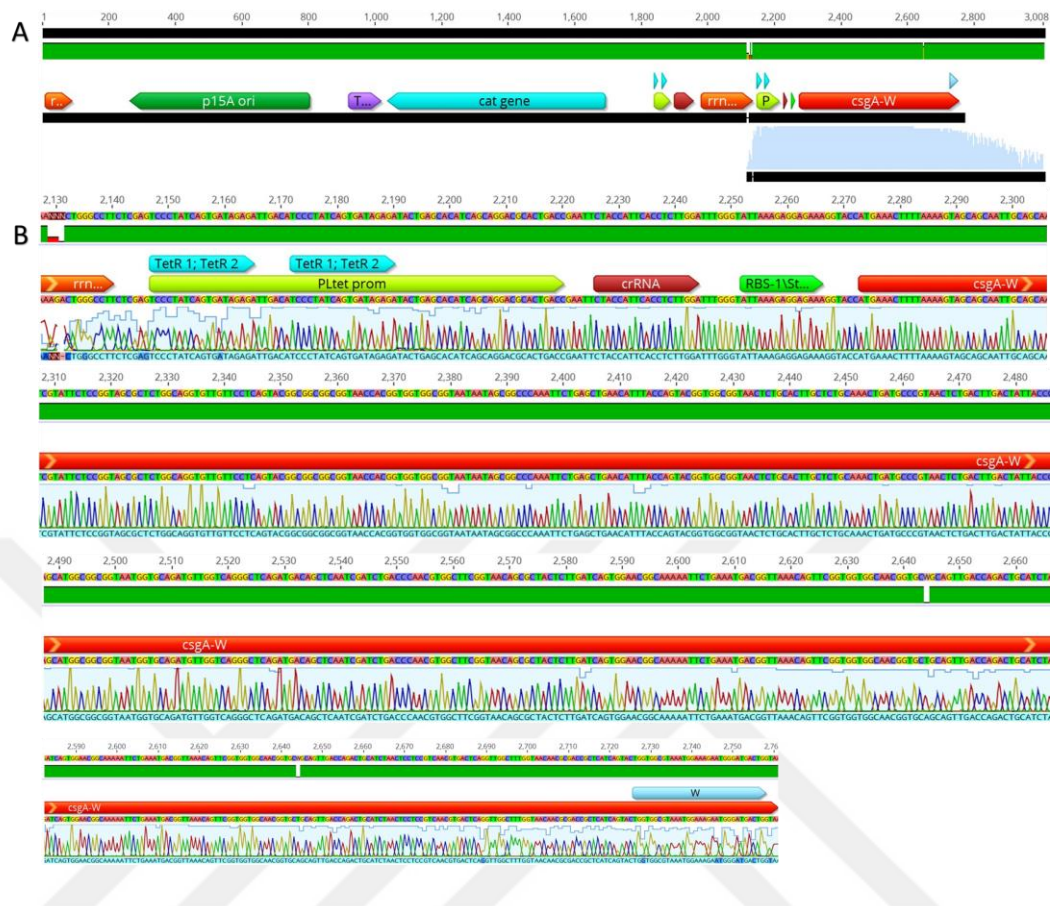


Figure D.6: Sanger DNA sequencing results of *csgA-W* in pZa vector. A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the green bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.

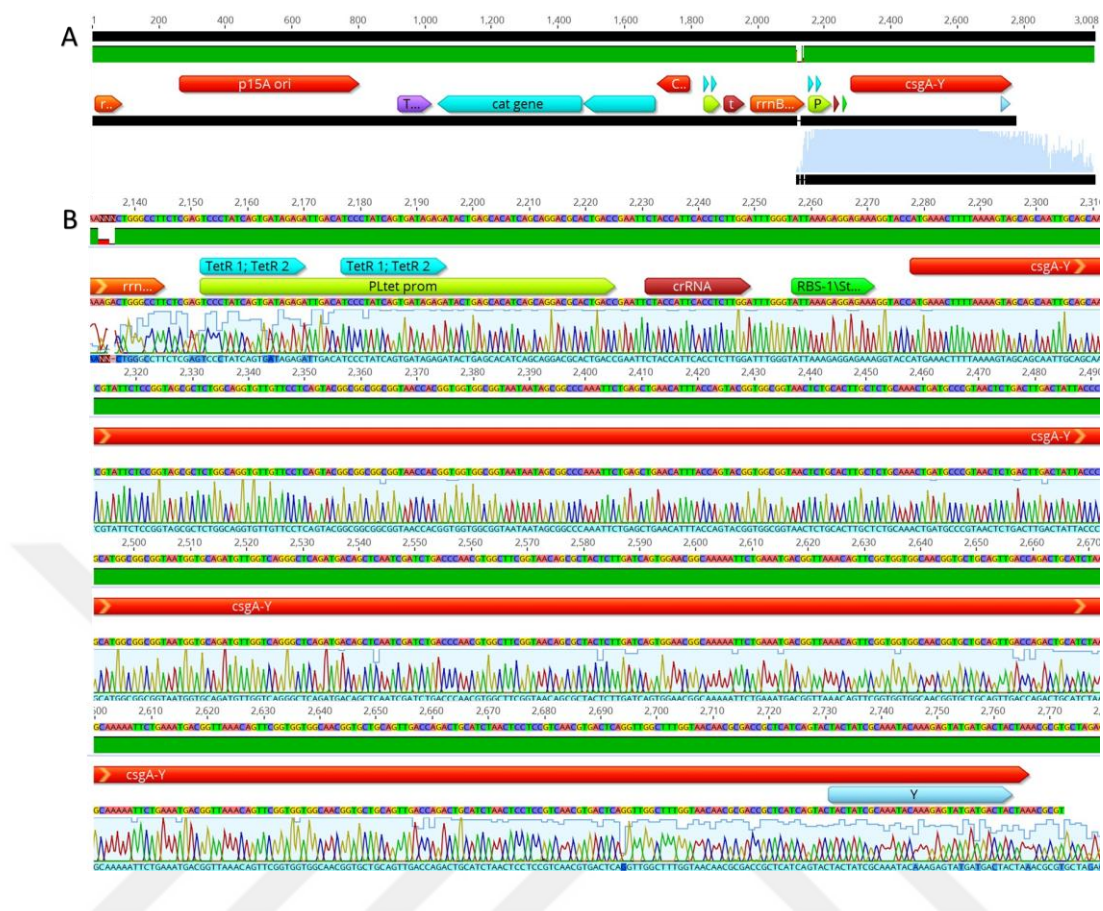


Figure D.7: Sanger DNA sequencing results of *csgAY* in pZa vector. A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the green bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.

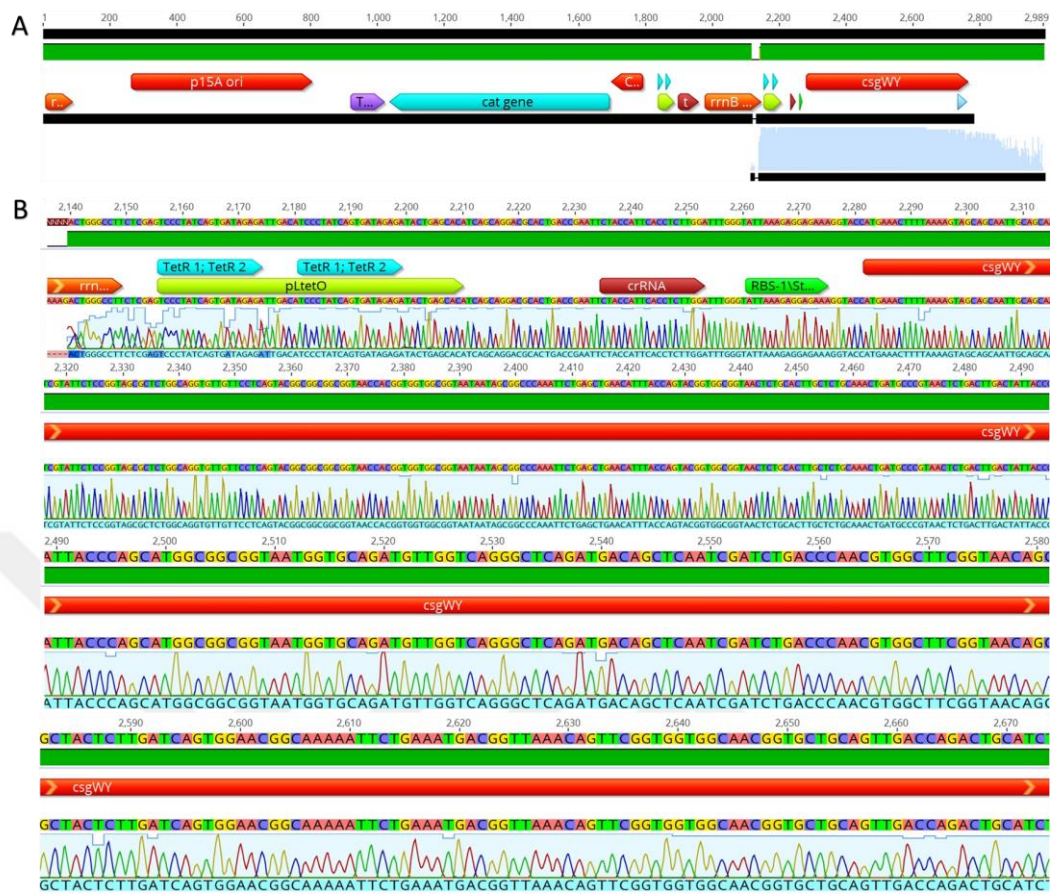


Figure D.8: Sanger DNA sequencing results of *csgAWY* in pZa vector. A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the green bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.

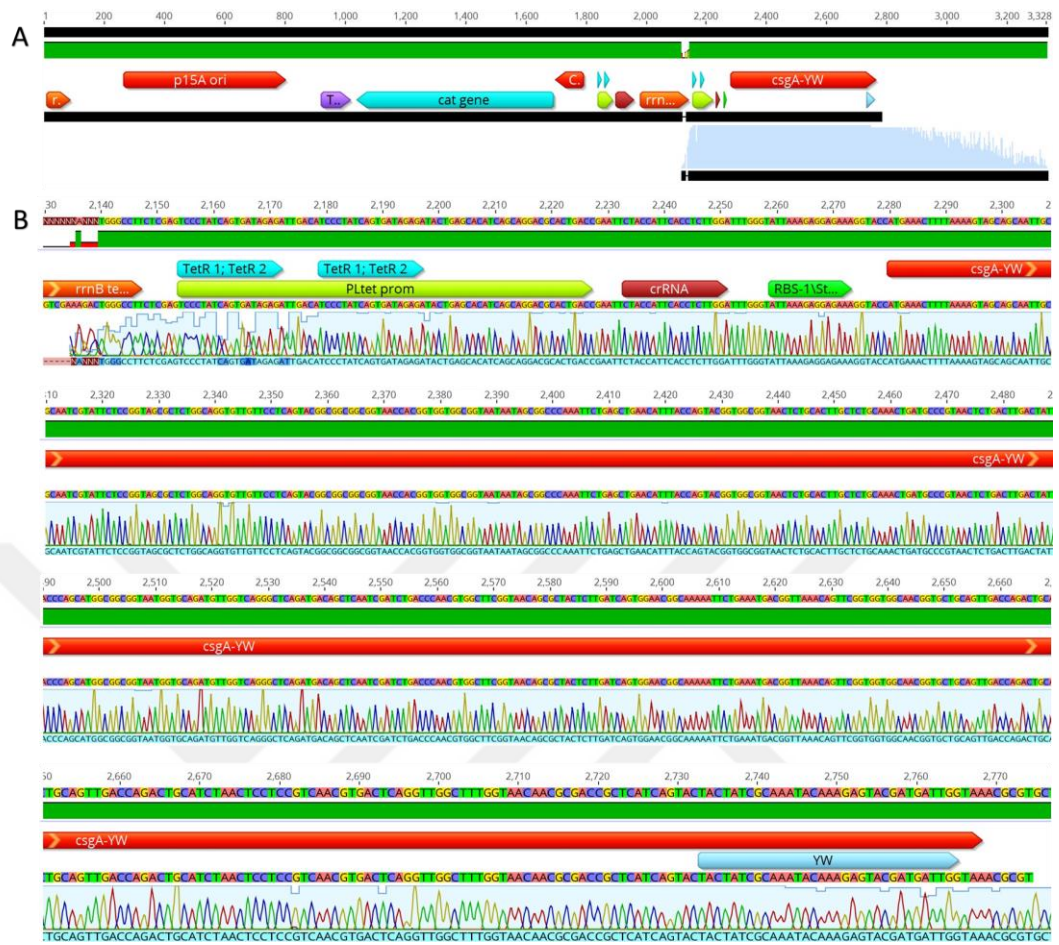


Figure D.9: Sanger DNA sequencing results of *csgAYW* in pZa vector. A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the green bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.

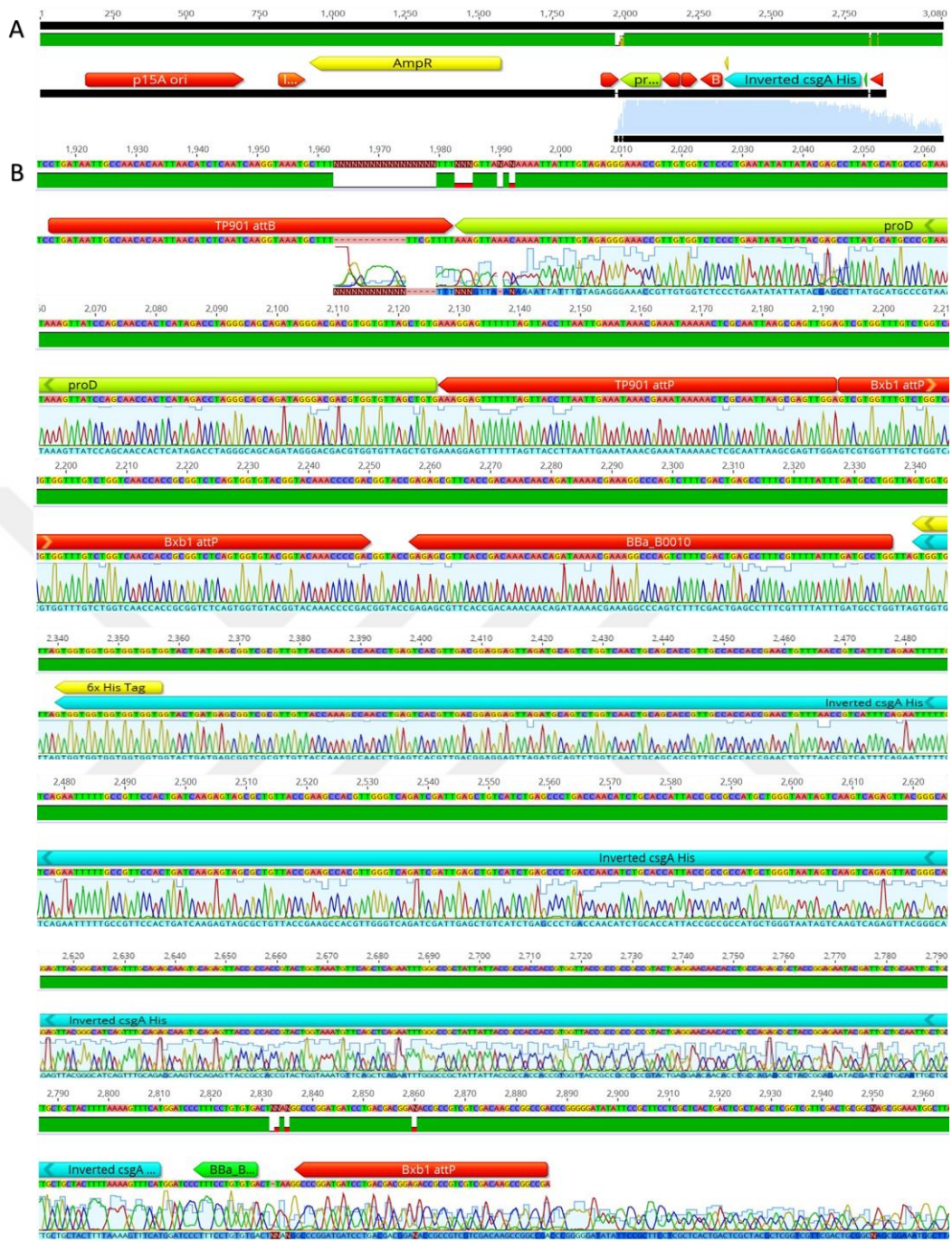


Figure D.11: Sanger DNA sequencing results of AND GATE csgAH in pZa vector. A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the green bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.



Figure D.12: Sanger DNA sequencing results of OR GATE csgAH in pZa vector.

A) Sequence chromatogram was compared with the reference designs for each of the genetic construct. Continuity of the green bar signifies nucleotide-level match between design and actual sequence. B) Close-up view shows the nucleotides and chromatogram in detail.

APPENDIX E

Force Spectroscopy Experiments

For a given sample, at least 20 force vs. distance (FD) curves are collected, as designated by the red points in Figure E1(a). The FD curve extracted for each indentation point can be divided into two parts (Figure E1(b)): The first part involves the region where the tip is away from the surface, and as such, the interaction force between the tip and the sample is recorded as zero. The second part of the FD curve starts once the tip makes contact with the surface, spotted as an abrupt positive change in the slope of the FD curve at a piezo displacement defined as ~ 0 nm. The interaction force thereafter increases more or less linearly with increasing piezo displacement for all investigated samples.

As the first step toward extracting the overall Young's modulus value for a given sample, for each experimentally recorded FD curve, the slope (or in other words, the “equivalent” spring constant, k_{eq}) is determined as shown in Figure E1(b). It should be taken into account that the extracted k_{eq} value corresponds to the “combined resistance to deformation” involving the stiffness (i.e., spring constant) values associated with the (i) AFM cantilever (k_{cant}) and (ii) the amyloid nanofiber (k_{fiber}) on which the FD experiment is performed, as shown by the “springs in series” model of Figure E1(c). Since k_{cant} is known thanks to the calibration step described in Appendix F, k_{fiber} can be calculated in a straightforward fashion from the measured k_{eq} values. While k_{fiber} values associated with different samples can be contrasted with each other to perform a

comparison of overall stiffness, following the steps outlined below, *intrinsic* Young's modulus values for each amyloid nanofiber sample can be extracted.

Following the determination of the k_{fiber} value for a given FD curve, two additional curves describing the change of the cantilever “deflection” (which is directly proportional to the force measured by the cantilever through $F = k_{cant} * d$) with increasing piezo displacement (Z) are drawn: One on the stiff mica substrate (d_{mica} vs. Z) and one on the amyloid nanofiber (d_{fiber} vs. Z), as in Figure E1(d). As mica is considered to be infinitely stiff when compared to the amyloid nanofiber, it will not deform during the FD experiment and all piezo displacement will be reflected in the cantilever deflection such that $d_{mica} = Z$; in other words, the slope of the deflection vs. piezo displacement curve would be 1. On the other hand, for the amyloid nanofibers, the recorded slope for the deflection vs. piezo displacement curves will always be smaller than 1 (and in fact, equal to $\frac{k_{eq}}{k_{cant}}$) since part of the piezo displacement will be taken up by the deformation of the sample.

Subsequently, using the two deflection curves shown in Figure E1(d), the “indentation” values (δ) associated with the deformation of the nanofibers at each piezo displacement Z are calculated as $\delta = d_{mica} - d_{fiber}$. In a second step, the δ values at each piezo displacement are plotted against the interaction force F recorded at the same piezo displacement as in Figure E1(e) such that a “force vs. indentation” curve is obtained. While the majority of FD experiments performed on biological materials in the literature result in non-linear force vs. indentation curves, we have exclusively observed a linear relationship between force and

indentation in our experiments. Considering that the tip apex in our experiments is blunt-ended (see Appendix F) and that Hertzian contact between a flat-ended cylindrical punch and a flat, elastic half-space is expected to result in a linear force vs. indentation behaviour according to the equation $F = 2RE^*\delta$ (where E^* is the *effective* Young's modulus), this result is perhaps not surprising. Finally, the Young's modulus for the amyloid nanofiber (E_{fiber}) is found via the following relationship:

$$E_{fiber} = \frac{1 - \nu_{fiber}^2}{\frac{1}{E^*} - \frac{1 - \nu_{tip}^2}{E_{tip}}}$$

where the Poisson's ratio of the tip (ν_{tip}) and the fibers (ν_{fiber}) are assumed to be 0.3, and the Young's modulus for the silicon tip (E_{tip}) is taken as 130 GPa.

Generally, to study the deformation-dependent mechanical behavior of the samples through force spectroscopy, each curve is divided into “segments” defined by piezo displacements that start at 1 nm and progressively increase by 1 nm until a segment length of 50 nm is reached.

To limit the influence of the stiff substrate (mica) on the measured mechanical behavior of the fibers, this procedure is stopped for each curve at piezo displacements that result in indentations equal to ~75% of the fiber height (so that the FD curves resulting in indentations beyond this criterion are not considered while calculating the Young's modulus values). For each segment, mean and standard variation values for Young's modulus are determined by taking into account all FD curves acquired on that sample, as described above. In other

words, the procedure described above (that results in the calculation of E_{fiber}) is repeated for each segment.

Finally, to obtain values reflecting the overall mechanical behavior of a sample over a range of deformations, mean and standard error of mean (SEM) values for Young's Modulus are calculated by taking into account all applicable segments (from 1 nm up to 50 nm). These are the values reported in Figure 4.7.

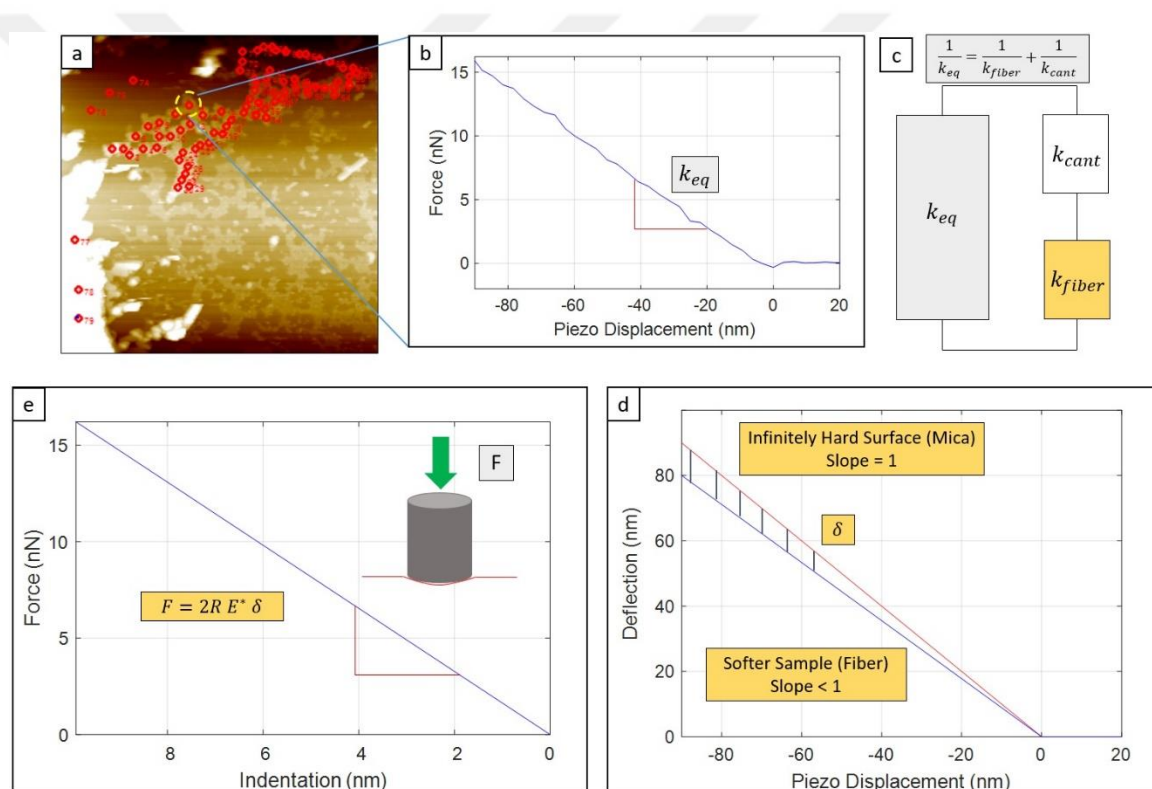


Figure E1. The procedure for extracting effective Young's modulus values (E^*) from the processing of FD curves. (a) Topographical AFM image acquired on sample CsgAM-B with red points indicating positions where FD curves were taken. Please note that several FD curves have been acquired on cells and the mica substrate for reference. (b) A representative FD curve acquired on the sample surface, featuring linear evolution of

force with piezo displacement, where the slope is determined as k_{eq} . (c) The “springs in series” model depicting the relationship between the measured stiffness (k_{eq}) and the spring constant values of the cantilever (k_{cant}) and the nanofiber (k_{fiber}). (d) Cantilever deflection vs. piezo displacement curves on mica (i.e., infinitely hard substrate) and the nanofiber, from which indentation values (δ) are calculated. (e) The corresponding force vs. indentation curve which is of linear character. The effective Young’s modulus value (E^*) is found by employing the equation in the inset relating force and indentation for a Hertzian contact between a flat-ended cylindrical punch and a flat, elastic half-space.

APPENDIX F

Cantilever Spring Constant Calibration and AFM Tip Apex Characterization

A single probe (Nanosensors PPP-CONTR) has been used for all AFM measurements presented in this work. The proper calibration of the normal spring constant of the cantilever (k_{cant}) as well as the structural characterization of the tip apex (to find the equivalent tip radius, R) are crucial for extracting accurate Young’s modulus values from force spectroscopy experiments.

k_{cant} is estimated using the well-known Sader’s method [262] utilizing the resonance frequency ($f_{res} = 13,688$ Hz) and the nominal dimensions ($l = 450$ μm , $w = 50$ μm and $t = 2$ μm) of the cantilever, whereby a $\sim 2\%$ reduction in the resonance frequency due to damping in air (as compared to vacuum) as well as the existence of a 30-nm-thick reflective aluminum layer are also taken into account, resulting in $k_{cant} = 0.20$ N/m.

R can be estimated using scanning electron microscopy (SEM) imaging, as shown in Figure F1. The SEM images shown in Figure F1 reveal a mostly *blunt-ended* tip apex which can be approximated as a cylindrical *flat punch* with an equivalent radius of $R \approx 75$ nm. As shown in Appendix E, this approximation is in alignment with *linear* force-indentation curves experimentally obtained on the amyloid nanofibers, which would be expected for Hertzian contact between a flat-ended cylindrical punch and a flat, elastic half-space.

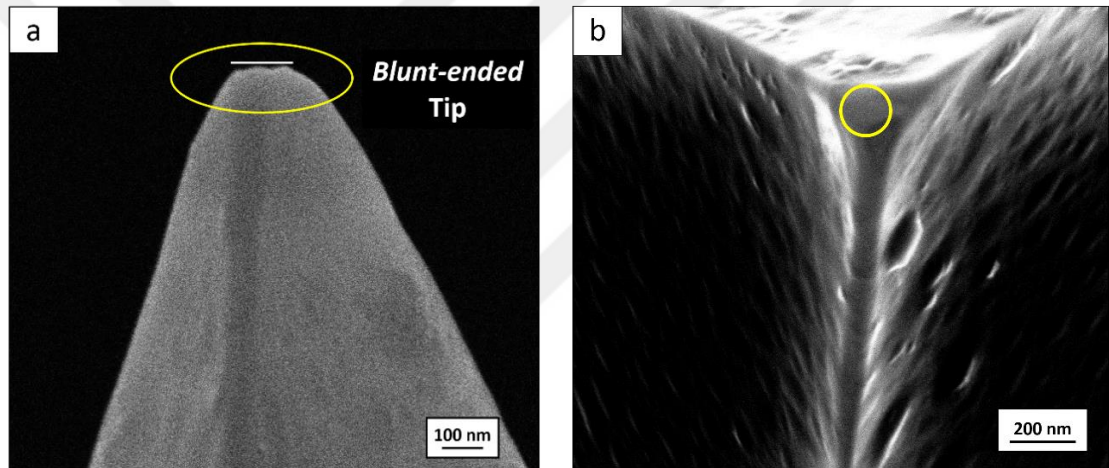


Figure F1. (a) Side-view and (b) top-view SEM images of the tip apex associated with the AFM cantilever used in the experiments.