Engineering thickness-controllable catalytic biofilms through synthetic biology approaches

Hu, Yidan

2019

Hu, Y. (2019). Engineering thickness-controllable catalytic biofilms through synthetic biology approaches. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/84132>

<https://doi.org/10.1021/bk-2019-1323>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**Engineering thickness-controllable catalytic biofilms
through synthetic biology approaches**

Hu Yidan

Interdisciplinary Graduate School

Singapore Centre for Environmental Life Sciences Engineering

**Engineering thickness-controllable catalytic biofilms through
synthetic biology approaches**

Hu Yidan

Interdisciplinary Graduate School

Singapore Centre for Environmental Life Sciences Engineering

A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirements for the degree of

Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

18/08/2019

.....
Date

.....
Hu Yidan

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

18/08/2019

.....
Date

.....
Cao Bin

Authorship Attribution Statement

This thesis work has been published in 1 book chapter and 2 papers where I was the first or co-first author. Part of the thesis work was also presented in one international conference and one workshop where I was the presenter.

Chapter 2 is published as Yidan Hu, Manisha Mukherjee and Bin Cao (2019) Biofilm biology-informed biofilm engineering for environmental biotechnology, in **Biofilm Engineering** (R. Navanietha Krishnaraj and Rajesh Sani, Eds), American Chemical Society (ACS) Publications.

The contributions of the co-authors are as follows:

- Dr. Manisha Mukherjee and I prepared the draft of the book chapter and we contributed equally to this publication.
- Assoc. Prof. Cao Bin was invited to contribute this book chapter to the edited book “Biofilm Engineering” by ACS Publications and revised the draft prepared by me and Dr. Manisha Mukherjee.

Chapter 3 is published as Yidan Hu, Yichao Wu, Manisha Mukherjee and Bin Cao (2017) A near-infrared light responsive c-di-GMP module-based AND logic gate in *Shewanella oneidensis*. **Chemical Communications** 10: 1646 -1648.

The contributions of the co-authors are as follows:

- Assoc. Prof. Cao Bin conceived the idea and revised the manuscript.

- I designed this study and analyzed data together with Assoc. Prof. Cao Bin.
- I conducted all the experiments and prepared the manuscript draft with the help of Dr. Wu Yichao and Dr. Manisha Mukherjee.
- Dr. Wu Yichao and Dr. Manisha Mukherjee contributed to the acquisition and analyses of the CLSM images.

Chapter 4 is published as as Yidan Hu, Xiaobo Liu, Aloysius Teng Min Ren, Ji-Dong Gu, Bin Cao (2019) Optogenetic modulation of a catalytic biofilm for biotransformation of indole into tryptophan. **ChemSusChem** 12: 1-8.

The contributions of the co-authors are as follows:

- Assoc. Prof. Cao Bin conceived the idea.
- Assoc. Prof. Cao Bin and Assoc. Prof. Gu Ji-Dong revised the manuscript.
- I designed this study and analyzed data together with Assoc. Prof. Cao Bin.
- I conducted all the experiments and prepared the manuscript draft with the help of Mr. Liu Xiaobo and Mr. Ren Aloysius.
- Mr. Liu Xiaobo and Mr. Ren Aloysius contributed to the conduction and analyses of biofilm-mediated biotransformation.

Chapter 5 is published as Manisha Mukherjee, Yidan Hu (co-first author), Chuan Hao Tan, Scott A. Rice, Bin Cao (2018) Engineering a light-responsive, quorum quenching biofilm to mitigate biofouling on water purification membranes. **Science Advances** 12: eaau1459.

The contributions of the co-authors are as follows:

- Assoc. Prof. Cao Bin conceived the idea and revised the manuscript.
- Dr. Manisha Mukherjee and I designed the experiments and analyzed data together with Assoc. Prof. Cao Bin. Dr. Manisha Mukherjee and I prepared the manuscript drafts.
- I conducted all molecular work including the design and construction of the dichromatic, optogenetic c-di-GMP and quorum quenching gene circuits. I also conducted the experiments for testing the performance of the light-responsive gene circuit under NIR light and blue light in the batch mode. Dr. Mukherjee conducted the experiments for testing the activity of the quorum quenching gene circuit and the performance of the engineered biofilms in membrane-based flow cells.
- Dr. Chuan Hao Tan and Assoc. Prof. Scott A. Rice assisted in the design of the quorum quenching gene circuit and helped revise the manuscript.

[Oral Presentation] Hu Y, Cao B, Engineering light-modulated biofilms through synthetic biology approaches, **Essex Synthetic Biology School (Workshop)**, University of Essex, UK, Jul 2-6, 2018.

- I prepared the presentation slides based on part of my thesis work and Assoc. Prof. Cao Bin revised the slides. I presented the slides in the workshop.

[Poster Presentation] Hu Y, Manisha Mukherjee, Cao B, Engineering a light-responsive, quorum quenching biofilm to mitigate biofouling on water purification

membrane, **9th ICBE – International Conference on Biomolecular Engineering**,
Newport Beach, CA, USA, Jan 6-9, 2019.

- I prepared the poster based on part of my thesis work together with Dr. Manisha Mukherjee. Assoc. Prof. Cao Bin revised the poster. I presented the poster in the conference.

18/08/2019

.....
Date

.....
Hu Yidan

Acknowledgements

Upon the completion of the thesis, I would like to take this opportunity to express my sincere gratitude to all those who helped me during my PhD study. Without their support, assistance, advice and guidance, the completion of my thesis would not have been possible.

I would like to gratefully acknowledge the mentorship and guidance of my supervisor, Assoc. Prof. Cao Bin. He provided effective supervision and training that have helped me develop a future career in academia. I am deeply inspired by his enthusiasm for scientific research, rigorous scientific attitude and critical thinking skills, which are valuable treasure for my future academic career. I also appreciate his patience, encouragement, and professional advices during my PhD study. I am immensely fortunate to have such a great supervisor guiding me through this challenging but precious journey.

I would like to thank thesis committee members Prof. Stefan Wuertz, Assoc. Prof. Yang Liang and Assoc. Prof. Liang Zhao-Xun. Their insightful comments and valuable suggestions have contributed greatly to my thesis work.

I am also greatly indebted to many young and talented scientists whose assistance have accelerated my research progress. I am grateful to Dr. Wu Yichao and Dr. Manisha Mukherjee for their guidance on experimental biofilm studies; Mr. Liu Xiaobo, Mr. Aloysius Teng and Mr. Sakcham Bairoliya for their help in conducting some of experiments; Ms. Azean Zaiden, Dr. Daphne Ng, Dr. Amit Kumar and Dr. Tan Shi Ming for their suggestions on the revision of manuscripts, thesis draft and presentation slides.

Although earning a doctorate degree is an academic journey with many challenges, these lovely group members have made my journey very joyful.

My gratitude also extends to my family. I am immensely fortunate to have the full support of my husband, Mr. Adnan Ali Khan, and my parents. This journey would not have been possible without your support, company and understanding. I am not used to saying this out, but I love you all!

Last but not the least, I would like to thank the Ministry of Education, Nanyang Technological University, Interdisciplinary Graduate School and Singapore Centre for Environmental Life Sciences Engineering for the PhD scholarship awarded to me.

Table of Content

Statement of Originality	i
Supervisor Declaration Statement.....	ii
Authorship Attribution Statement	iii
Table of Content.....	ix
Abstract.....	xiii
List of tables.....	xv
List of figures.....	xvi
List of abbreviations	xxiii
Chapter 1. Introduction.....	1
1.1 Research background and problem statement.....	1
1.2 Objective	2
1.3 Thesis outline	2
Chapter 2. Literature review	5
2.1 Introduction	5
2.2 Biofilm biology.....	6
2.2.1 Biofilm life cycle.....	6
2.2.2 Biofilm matrix	10
2.2.3 Chemical signaling systems in biofilm development.....	12
2.3 Biofilm-mediated bioprocess	18
2.3.1 Biofilms-mediated bioprocess for viroous applications	18
2.3.2 Role of biofilm thickness in biofilm-mediated bioprocesses	20
2.4 Biofilm engineering	22

2.4.1 C-di-GMP-targeted biofilm engineering	22
2.4.2 QS-targeted biofilm engineering	25
2.5 Summary	27
Chapter 3. A near-infrared light responsive c-di-GMP module-based AND logic gate in <i>Shewanella oneidensis</i>.....	30
3.1 Introduction	30
3.2 Materials and methods.....	32
3.2.1 Bacterial strains and growth conditions	32
3.2.2 Gene circuit construction and transformation	34
3.2.4 Confocal laser scanning microscopy (CLSM) and image analysis	39
3.2.5 MFC set up	39
3.2.6 The measurement of MFC power density	40
3.2.7 Electrode-attached biofilm images and biomass measurement.....	40
3.3 Results and discussion.....	41
3.3.1 Design of the genetic circuits	41
3.3.2 Verification of the genetic circuits by static biofilm assay	42
3.3.3 Biofilm-based AND logic in MFC	45
3.4 Conclusion.....	49
Chapter 4. Optogenetic modulation of a catalytic biofilm for biotransformation of indole into tryptophan	50
4.1 Introduction	50
4.2 Materials and methods.....	52
4.2.1 Plasmids, bacterial strains and growth conditions.....	52
4.2.2 Congo red assay.....	53
4.2.3 Biofilm formation assay	53
4.2.4 CLSM and image analyses	54

4.2.5 Biotransformation of indole to tryptophan by planktonic cells.....	54
4.2.6 Biotransformation of indole to tryptophan by biofilms.....	55
4.2.7 Quantification of indole, tryptophan and ammonium	55
4.3 Results and discussion.....	56
4.3.1 The design of NIR light responsive, tryptophan-producing biofilm	56
4.3.2 Activity of NIR light responsive c-di-GMP gene circuit	60
4.3.3 Biotransformation catalyzed by planktonic cells	62
4.3.4 Biofilm-mediated biotransformation in submerged biofilm reactors.....	65
4.4 Conclusion.....	73
Chapter 5. Engineering a light-responsive, quorum quenching biofilm to mitigate biofouling on water purification membranes	75
5.1 Introduction	75
5.2 Materials and methods.....	77
5.2.1 Bacterial strains and culture conditions.....	77
5.2.2 Plasmid construction and transformation	78
5.2.3 Congo red assay.....	82
5.2.4 Biofilm formation assay	82
5.2.5 AHL degradation assay	83
5.2.6 Extraction and quantification of c-di-GMP	83
5.2.7 Dynamics of the light-responsive biofilm on FO membrane	84
5.2.8 Confocal microscopy and imaging.....	85
5.3 Results and discussion.....	86
5.3.1 Design of the engineering gene circuits	86
5.3.2 Light-controllable development of the engineered <i>E. coli</i> biofilm	89
5.3.3 QQ activity of the engineered <i>E. coli</i> biofilm and inhibitory effect on biofilm formation by <i>P. stewartii</i>	95

5.3.4 Light-modulated biofouling dynamics on FO membranes in membrane flow cells.....	99
5.4 Conclusion.....	105
Chapter 6. Summary and Recommendations.....	106
6.1 Summary	106
6.2 Recommendations for future research.....	108
6.2.1 Optimize the activity of the c-di-GMP synthase and hydrolase in the light-responsive gene circuit	108
6.2.2 Construct bacterial chassis with a low native c-di-GMP level for the improvement of the c-di-GMP gene circuit performance	109
6.2.3 Engineer a biofilm with a self-regulated c-di-GMP level.	110
References.....	112

Abstract

Biofilm represents the prevalent mode of microbial growth in natural, engineered and medical settings. Because of their natural immobilization and high tolerance to physicochemical stresses, biofilms have been demonstrated in recent studies as promising biocatalysts. Biofilm thickness is an important factor influencing solute diffusion and biocatalytic activity. It plays an important role in determining the overall performance of biofilm-mediated bioprocesses. Although biofilm development can be modulated through adjusting physicochemical parameters in biofilm-based bioreactors, it is highly challenging to maintain biofilm thickness in an optimal range allowing for sufficient activity and efficient mass transfer. The objective of this study is to develop novel strategies to control the thickness of biofilms through synthetic biology approaches for various biotechnological applications.

Biofilm development is often regulated by sophisticated intracellular signaling networks that modulate the levels of small molecules. Among them, Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) serves as a prevalent intracellular messenger that regulates biofilm formation. Recent studies modulated biofilm formation by constitutive or chemical-inducible (over)expression of c-di-GMP synthases or hydrolases. However, this may result in an overly thick or thin biofilm due to the irreversibility of constitutively expressing enzymes as well as limited spatial and temporal resolution of chemicals as inducers. Therefore, optogenetic tools are ideal for controlling biofilms, as light is non-invasive, easily controllable and cost-efficient.

In Chapter 3 of this thesis, I constructed and tested a near-infrared (NIR) light responsive c-di-GMP module for the modulation of an electroactive biofilm development by expressing a NIR responsive c-di-GMP synthase, BphS, in *Shewanella oneidensis*. In the study, NIR light increased c-di-GMP levels and enhanced biofilm formation on electrodes, resulting in increased bioelectricity generation in microbial fuel cells. In Chapter 4 of this thesis, I explored the use of optogenetic modulation of a catalytic biofilm to enhance biotransformation of indole into tryptophan by co-transforming the NIR light responsive c-di-GMP module and a tryptophan synthase gene circuit into *Escherichia coli*. In submerged biofilm reactors, NIR light improved biofilm formation to result in a ~ 30% increase in tryptophan yield. These results demonstrated the feasibility of applying light to “guide” the biofilm formation and improve catalytic performance in bioreactors.

As a constantly high c-di-GMP level could result in an undesirably thick biofilm, I subsequently introduced a blue light activated c-di-GMP hydrolase gene, *ebf*, into the NIR light responsive c-di-GMP module to bi-directionally control c-di-GMP levels and applied this dichromatic optogenetic c-di-GMP gene circuit to mitigate biofouling on water purification membranes in Chapter 5. In this study, quorum quenching activity was used to show the effectiveness of the light-responsive gene circuit to modulate biofilm thickness while exhibiting desirable functions. Taken together, this thesis work exemplifies the potential for translational research from biofilm biology to biofilm engineering for applied and environmental biotechnology.

List of tables

Table 3.1 Bacterial strains and plasmids used in this study	33
Table 3.2 Culture medium and buffer solution preparation.	33
Table 3.3 The primers used in this study	37
Table 3.4 Sequence of NIR light responsive c-di-GMP module.....	37
Table 5.1 Bacterial strains and plasmids used in this study	78
Table 5.2 Sequence of gene circuits	80

List of figures

Figure 1.1 Schematic illustration of the research design and key findings in Chapters 3 to 5.....	3
Figure 2.1 The biofilm life cycle. (1) Free swimming bacteria (2) attach to available surfaces. (3) The division of bacteria allows them to stick together and form small microcolonies. (4) With increasing size and density of aggregates, self-produced components are excreted to form the extracellular polymeric substance matrix for maintaining the function and structure of the biofilm. (5) When the biofilm become mature, gradients of factors (e.g., nutrient and oxygen) arise in the biofilm matrix, resulting in the heterogeneous nature of the biofilm. (6) In response to the change of environmental cues, cells “escape” from the matrix. Reproduced with permission from (McDougald et al., 2011) Copyright (2018) Springer Nature.	7
Figure 2.2 The biofilm matrix. (A) A model of biofilm formed on a solid surface. (B) The matrix mainly comprises of proteins, DNA and polysaccharides. Adapted with permission from (Flemming and Wingender, 2010) Copyright (2018) Springer Nature.....	11
Figure 2.3 Structure and physiological functions of c-di-GMP. The cellular level of c-di-GMP is regulated by DGCs with GGDEF domains and specific PDEs with EAL or HD-GYP domains. The c-di-GMP signaling networks coordinate many biological processes, including motility, virulence, biofilm formation and cell cycle. Adapted with permission from (Hengge, 2009) Copyright (2018) Springer Nature.	14
Figure 2.4 Typical quorum sensing circuits: (A) A Gram-negative one-component and (B) two-component QS system; (C) A Gram-positive one-component and (D) two-component QS system. The figure is prepared based on the studies (Miller, 2001; Waters and Bassler, 2005).	16
Figure 2.5 Relative effective diffusion coefficients of selected solutes in biofilms. D_e/D_{aq} is the relative effective diffusion coefficient, where D_e and D_{aq} are the effective diffusion coefficients in the biofilm and in water, respectively. MW represents molecular weight. Adapted from (Stewart, 2003).	21

Figure 2.6 Schematic illustration of the biofilm life cycle and potential targets for biofilm engineering.....28

Figure 3.1 The illustration for BioBrick (A) and the procedure of PYYDT-L construction (B). (A) The structure of a BioBrick. It consists of a BioBrick part and a plasmid backbone. BioBrick parts are functional DNA such as promoters, RBSs, Coding sequences, or terminators. BioBrick parts are flanked by an upstream prefix (including EcoRI and XbaI) and a downstream suffix (including SpeI and PstI) for convenient and fast assembly. (B) The procedure of PYYDT-L construction. IPTG inducible promoter *Ptac* and double terminator (shown by TT in plasmid map) were included in plasmid pYYDT. The gene *bphS* and *bphO* as an operon was synthesized *in vitro* and designed as a BioBrick part. Promoter *Ptac* to be placed in the front was cut by SpeI and SbfI, and *bphS-bphO* operon to be placed in the back was cut by XbaI and PstI. The two cut fragments were ligated and formed a long fragment containing *Ptac* and the synthetic operon still in BioBrick format, as the sticky ends of XbaI and SpeI are compatible. Due to PstI cutting site in pYYDT, SbfI was utilized to replace PstI as their sticky ends are compatible. The *bphS-bphO* operon was inserted into pYYDT, which are named as pYYDT-L. PYYDT-L contains three key elements, IPTG inducible promoter *Ptac*, diguanylate cyclase gene *bphS* and heme oxygenase gene *bphO*. Blue circles represent the RBSs. Sufficient translation of key components in the gene circuit was ensured by the strong RBS1 in front of *bphS* and *bphO*.34

Figure 3.2 (A) Schematic illustration of NIR light responsive c-di-GMP module based-AND logic gate in MFC. (B) Truth table of the AND logic gate.42

Figure 3.3 The performance of NIR light responsive c-di-GMP module for biofilm formation upon the induction of IPTG with different concentrations (A) and NIR light with different intensities (B). *** $P < 0.0005$, Student's t test.43

Figure 3.4 The performance of AND logic gate gene circuit for biofilm formation. (A) CLSM images of the static biofilm of *S. oneidensis* harbouring the constructed gene circuit and (B) the empty vector. (C) Biofilm biovolume per unit area of the bottom of each well and (D) total biofilm biomass (OD_{550nm}) in each well. *S. oneidensis* harboring the constructed gene circuit and the empty vector (E, F) was incubated for 24 h under four

input conditions: (0, 0), no IPTG or NIR light; (1, 0), only IPTG; (0, 1), only NIR light; and (1, 1), IPTG and NIR light. Experiments were conducted in triplicates. The scale bar represents 20 μm . * $P < 0.05$, ** $P < 0.005$, Student's t test.44

Figure 3.5 The performance of AND logic gate gene circuit for biofilm formation on the electrodes in MFC system. (A) CLSM images of biofilms and (B) The attached biomass (in terms of total proteins) of *S. oneidensis* harboring the constructed gene circuit on the anodes of AND logic gate-controlled MFCs. MFCs experiments were conducted in triplicates for each condition. Images were taken at three random spots on each electrode, and the representative image is shown here. The scale bar represents 50 μm . ** $P < 0.005$, Student's t test.46

Figure 3.6 The performance of the AND logic gate gene circuit in MFCs. (A) and (D) Polarization curves, (B) and (E) power density curves, and (C) and (F) maximum output power densities of the MFCs inoculated with the strain harbouring the AND-gated gene circuit or the control strain, respectively, under conditions with different inputs: (0, 0), no IPTG or NIR light; (1, 0), only IPTG; (0, 1), only NIR light; and (1, 1), IPTG and NIR light. The polarization curves were measured by recording the voltage output at various external resistances (200k–1k Ω). The current density and power density were calculated respectively using the formulae $I = V/(R \cdot S)$ and $P = VI$, where V , R and S represent voltage (V), resistance (R) and carbon cloth area (S) respectively. *** $P < 0.0005$, Student's t test.47

Figure 4.1 The design of NIR light responsive, catalytic *E. coli* biofilm for biotransformation of indole into tryptophan. The pYYDT-L vector with the pBBR1 origin and Km antibiotic resistance was employed to express NIR light responsive c-di-GMP synthase, while the pSTB7 vector with the pBR322 origin and Amp antibiotic resistance was used to overexpress tryptophan synthase. The c-di-GMP gene circuit was used to modulate biofilm formation. The tryptophan synthase TrpBA from *S. enterica* catalyzes the biotransformation of indole and serine into tryptophan in the presence of a cofactor PLP.....58

Figure 4.2 Tryptophan synthase mediated conversion of serine and (halo)indole (where X = F, Cl, Br) to (halo)tryptophan with PLP. PLP with serine yields Intermediate 1 by a

dehydration process mediated the enzyme. Then, intermediate 2 is resulted from the nucleophilic attack of indole on intermediate 1, and a subsequent hydrolysis releases the product halotryptophan and the cofactor PLP.59

Figure 4.3 Activity assay of the NIR light responsive c-di-GMP gene circuit. (A) Colony morphology on Congo red agar plates. (B) Representative CLSM images of the biofilms and (C) estimated biofilm biovolume of BL21/(pSTB7+pYYDT) and BL21/(pSTB7+pYYDT-L) formed on the sponges after 36 h incubation. Experiments were conducted in triplicate, and for each replicate, three representative images were acquired and quantified. The scale bar represents 50 μ m. **** P < 0.0001, Student's *t* test.....61

Figure 4.4 Activity assay of NIR light responsive c-di-GMP module using 50% LB medium. (A) Confocal images of biofilm and (B) biofilm biovolume of BL21/(pSTB7+pYYDT) and BL21/(pSTB7+pYYDT-L) on sponges under dark or NIR light. The scale bar represents 50 μ m. **** P < 0.0001, Student's *t* test.62

Figure 4.5 Growth curves of BL21/(pSTB7+pYYDT-L) grown (A) in M9 medium or (B) in 50% LB medium in the presence and absence of NIR light.63

Figure 4.6 Biotransformation of indole into tryptophan using planktonic cells of the engineered strain BL21/(pSTB7+pYYDT-L). The cells constitutively express tryptophan synthase and the NIR light responsive c-di-GMP synthase. Concentration profiles of indole and tryptophan in the reaction solutions: (A) Cells pre-grown in M9 media and used as biocatalysts under dark condition; (B) Cells pre-grown in M9 media and used as biocatalysts under NIR light; (C) Cells pre-grown in 50% LB media and used as biocatalysts under dark condition; (D) Cells pre-grown in 50% LB media and used as biocatalysts under NIR light. Ammonium concentration profiles also shown in (C) and (D). Ammonium undetectable for conditions (A) and (B). Experiments were conducted in triplicate.64

Figure 4.7 Percentage of indole depletion by BL21/(pSTB7+pYYDT-L) grown in M9 medium in the presence and absence of NIR light (1.33 mW/cm²).65

Figure 4.8 Effect of NIR light on biofilms in submerged biofilm reactors: (A) Representative CLSM images of biofilms, (B) estimated thickness and cell viability of

biofilms in the presence and absence of NIR light. The scale bar represents 50 μm . (C) Tryptophan concentration in effluent from the reactor under NIR light and in the dark. **** $P < 0.0001$, Student's t test.67

Figure 4.9 Cell detachment rate from biofilms in the absence and presence of NIR light before and after 6-h exposure to indole. * $P < 0.05$, Student's t test.68

Figure 4.10 Biotransformation of indole into tryptophan by NIR light responsive biofilm in submerged biofilm reactors continuously supplied with M9 medium: (A) Representative CLSM images of the biofilms, (B) estimated biofilm thickness, (C) tryptophan concentration in effluent, (D) tryptophan concentration normalized to the biofilm thickness, and (E) the amount of tryptophan produced by the biofilms in the presence and absence of NIR light. The scale bar represents 50 μm . ** $P < 0.01$ and *** $P < 0.001$, Student's t test.71

Figure 4.11 (A) and (B) The amount of tryptophan produced by the biofilms in the presence and absence of NIR light when the concentrations of indole are 2 mM and 4 mM, respectively.73

Figure 5.1 Schematic illustration of the gene circuits in engineered *E. coli*. Dichromatic optogenetic module for regulating intracellular c-di-GMP levels comprising of a NIR light regulated DGC, BphS, and a blue light activated PDE, EB1. Quorum quenching gene circuit for producing AiiO, to reduce AHL regulated biofilm formation by other bacteria. A circle in front of each gene indicates a RBS; a T-sign at the end of the operon indicates a transcription terminator.88

Figure 5.2 The performance of the light-responsive gene circuit under NIR light and blue light. A) c-di-GMP-dependent curli fimbriae synthesis in *E. coli*. The control strain *E. coli*/pYYDT and the light-responsive strain *E. coli*/pYYDT-RB were grown on Congo red plates for 24 h at 25 °C in the dark or when exposed to NIR light or blue light. (B) Total biofilm biomass ($\text{OD}_{550\text{nm}}$) of the control strain *E. coli*/pYYDT and the light-responsive strain *E. coli*/pYYDT-RB in each well after incubation for 24 h at 25 °C, without shaking, under dark, NIR light and blue light. (C) Intracellular c-di-GMP concentration (pmol/mg protein) in the light-responsive strain *E. coli*/pYYDT-RB after incubation for 24 h at 25°C

in the dark or when exposed to NIR light or blue light. Experiments were conducted in triplicate. Error bar represents SD. $**P < 0.005$ and $***P < 0.0005$, Student's t test.89

Figure 5.3 Biofilm formation of the control *E. coli*/pYYDT and light-responsive *E. coli*/pYYDT-RB strains in biofilm flow cell. (A) CLSM images of biofilms at 24, 72 and 120 h. The red line represents biofilms formed under NIR light and the blue line represents biofilms formed under blue light. Each CLSM image contains one plan view and two sectional views of the biofilm. Scale bar, 20 μm . (B) Biofilm biovolume (expressed as biovolume per unit base area). The red and blue zones represent incubation under NIR light and blue light, respectively. Error bar represents the standard deviation.91

Figure 5.4 Biofouling dynamics of the control, *E. coli*/pYYDT, and light-responsive, *E. coli*/pYYDT-RB, strains on FO membrane in membrane flow cell. (A) The CLSM-compatible membrane flow cell for nondestructive monitoring of biofouling dynamics on membranes. The device is made of three independent channels that are divided by a flat sheet membrane into two different channels. (B) CLSM images of biofilms grown on the FO membrane at four different timepoints ($t = 48, 72, 96$ and 120 h). The blue line represents biofilms formed after 24 h of incubation under blue light, and the red line represents biofilms formed after 24 h of incubation under NIR light. Each CLSM image contains one plan view and two sectional views of the biofilm. Scale bar, 20 μm . (C) Biofilm biovolume (expressed as biovolume per unit base area) and (D) biofilm thickness. (E) Comparisons of permeate flux through membranes at different time points. The gray, blue, and red zones represent incubation in the dark and under blue light and NIR light, respectively. Experiments were conducted in triplicate and five representative images were acquired and quantified for each replicate. Error bar represents SD. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, Student's t test.95

Figure 5.5 AHL degradation is mediated by quorum quenching enzymatic activities. (A) Inactivation of exogenous 3OC6-HSL by the light-responsive QQ strain, *E. coli*/(pYYDT-RB + pAiiO), were assessed over 24 h at room temperature. Residual 3OC6-HSL was quantified using the *E. coli* JB525 bioassay at 0, 2, 6, 12 and 24 h post incubation. (B) Inactivation of exogenous 3OC6-HSL by *E. coli*/(pYYDT-RB + pAiiO) cells and the cell-free supernatant. (C) Inactivation of exogenous 3OC6-HSL by *E. coli*/(pYYDT-RB +

pAiiO) cells incubated in the dark, under NIR light and blue light. (D) CLSM images of biofilm formation by YFP tagged *P. stewartii* on preformed light-responsive QQ biofilm *E. coli*/(pYYDT-RB + pAiiO) and QQ control biofilm *E. coli*/(pYYDT-RB + pmAiiO) on biofilm flow cells after 24 h. Scale bar, 20 μ m. Experiments were conducted in triplicate.

.....97

Figure 5.6 Coculture biofilm formation by the mCHERRY-tagged control *E. coli* (QQ negative) and YFP-tagged *P. stewartii*. (A) CLSM image of co-culture biofilm at 24 h. Scale bar, 20 μ m. (B) Biofilm biovolume (expressed as biovolume per unit base area). $P > 0.1$, Student's *t* test.99

Figure 5.7 Biofouling dynamics of *P. stewartii* in the presence of the light-responsive QQ biofilm, *E. coli*/(pYYDT-RB + pAiiO), and a QQ control biofilm, *E. coli*/(pYYDT-RB + pmAiiO), in a membrane flow cell. (A) CLSM images of biofilms formed on the FO membrane at 24, 48, 72 and 96 h. The blue line represents imaging after 24 h of incubation under blue light, and the red line represents imaging after 24 h of incubation under NIR light. Each CLSM image contains one plan view and two sectional views of the biofilm. Scale bar, 20 μ m. (B) Total biofilm biovolume of the consortium (expressed as biovolume per unit base area). (C) Total biofilm thickness of the consortium. (D) Biofilm biovolume of *P. stewartii* in different consortium at 96 h. (E) Comparisons of permeate flux through membranes at different time points. The gray, blue, and red zones represent incubation in the dark or under blue light and NIR light, respectively. Error bar represents SD. $*P < 0.05$ and $**P < 0.005$, $***P < 0.0005$, Student's *t* test.101

List of abbreviations

c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
NIR	Near-infrared
EPS	Extracellular polymeric substances
DGCs	Diguanylate cyclases
PDEs	Phosphodiesterases
QS	Quorum sensing
AI	Autoinducer
AHL	N-acyl homoserine lactones
DCP	Dichlorophenol
DNT	Dinitrotoluene
QSIs	Quorum sensing inhibitors
QQ	Quorum quenching
FO	Forward osmosis
CRISPR	Clustered regularly interspaced short palindromic repeats
sRNA	Small regulatory RNA
MFC	Microbial fuel cell
IPTG	Isopropyl β -D-thiogalactoside
EET	Extracellular electron transfer
pGpG	5'-phosphoguanylyl-(3'-5')-guanosine
BV	Biliverdin
MPD	Maximum power density
DAP	Diaminopimelic acid
Amp	Ampicillin
Km	Kanamycin
Gm	Gentamycin
RBSs	Ribosome binding sites
KEGG	Kyoto Encyclopedia of Genes and Genomes
JCAT	Java Codon Adaptation Tool
PBS	Phosphate buffer solution
CLSM	Confocal laser scanning microscopy
PI	Propidium iodide
PLP	Pyridoxal 5-phosphate
DMAB	Dimethylaminobenzaldehyde
LB	Lysogeny broth
PCR	Polymerase chain reaction

Chapter 1. **Introduction**

1.1 Research background and problem statement

In natural and engineered environments, bacterial cells often attach onto surfaces or interfaces and develop into sessile communities known as biofilms. Cells in a biofilm matrix are embedded in self-produced extracellular polymeric substances (Flemming and Wingender , 2010; Flemming et al., 2016). Extensive studies have demonstrated a wide range of advantages of the biofilm mode of life. For example, compared to their planktonic counterparts, biofilms have been reported to be more tolerant to harsh physicochemical conditions generated by various toxic contaminants or biocides (Cao et al., 2012; Mah and O'Toole, 2001). Because of these advantages, biofilms have shown great promise for applications in environmental and industrial biotechnologies, such as bioremediation, biocatalysis and microbial fuel cells (Halan et al., 2012; Min and Logan, 2004; Singh et al., 2006).

The overall performance of a biofilm-mediated bioprocess is determined by many factors including biofilm thickness. The thickness of a biofilm greatly influences mass transfer processes in the biofilm and its overall biocatalytic activity. Thin biofilms are not able to provide sufficient catalytic activity, whereas overly thick biofilms may contain unproductive biomass at the base of the biofilm as a result of the poor diffusion of solutes (e.g. substrates and nutrients) (Stewart, 2003). Therefore, it is highly desirable to maintain the biofilm thickness within an optimal range that allows for sufficient activity and efficient mass transfer. Although biofilm development can be modulated via physicochemical

parameters in biofilm-based bioreactors, it is highly challenging to maintain biofilm thickness in an optimal range. Synthetic biology enables designing and controlling biological processes to achieve predictable, reprogrammable and novel performances, thus providing new opportunities to achieve controllable biofilms for the improvement of biofilm-mediated bioprocesses.

1.2 Objective

The objective of this study is to develop novel strategies for controlling the thickness of biofilms through synthetic biology approaches for various biotechnological applications. In this thesis, optogenetic approaches were developed to control biofilm dynamics because, compared to chemicals, light is less invasive and more cost effective. Specifically, this thesis aims to:

- (1) Construct and test NIR light responsive c-di-GMP module for the modulation of biofilm development.
- (2) Demonstrate the use of the c-di-GMP module to engineer a NIR light-responsive catalytic biofilm for chemical synthesis.
- (3) Achieve bidirectional control of biofilm thickness by constructing gene circuits that are responsive to NIR light and blue light and apply the light-controllable biofilm in environmental biotechnology.

1.3 Thesis outline

This thesis includes 6 chapters, which are highlighted as follows:

Chapters 1 and 2 provide the background, research gaps, objective and a summary of previous studies in biofilm biology and biofilm engineering, while Chapter 6 provides a summary of this thesis work and a brief description of future recommendations. Chapters 3 to 5 present my research work and the research design as well as key findings are shown in Figure 1.1.

Figure 1.1 Schematic illustration of the research design and key findings in Chapters 3 to 5.

In Chapter 3, a NIR light responsive c-di-GMP module was constructed and tested for the modulation of an electroactive biofilm in microbial fuel cells for bioelectricity generation. In Chapter 4, the compatibility of the NIR light responsive c-di-GMP module with other gene circuits was tested achieve a NIR light responsive catalytic biofilm for chemical synthesis.

In Chapter 5, a quorum quenching bacterial biofilm was engineered so that its growth and dispersal can be modulated by light through a dichromatic, optogenetic c-di-GMP gene circuit in which the bacterial cells sense NIR light and blue light to adjust its biofilm formation by regulating the c-di-GMP level. The potential application of the engineered light responsive, quorum quenching biofilm in mitigating biofouling of water purification forward osmosis (FO) membranes was also demonstrated.

Chapter 2. Literature review

*This chapter has been published as Yidan Hu, Manisha Mukherjee and Bin Cao (2019) Biofilm biology-informed biofilm engineering for environmental biotechnology, in **Biofilm Engineering** (R. Navanietha Krishnaraj and Rajesh Sani, Eds), **American Chemical Society (ACS) Publications**. Permission has been granted by the licensed content publisher “American Chemical Society” to use the published content as a chapter in this thesis.*

2.1 Introduction

Bacterial cells frequently adhere to each other and/or to a surface or interface, forming biofilms. In biofilms, cell aggregates are embedded and held together by a matrix of self-produced extracellular polymeric substances (EPS) (O'Toole et al., 2000). Biofilm is a ubiquitous mode of bacterial growth in most natural, medical and engineered systems (Hall-Stoodley et al., 2004). Previous studies have reported the formation of biofilms on human tissues and medical implants which often results in intractable chronic infections (Hatt and Rather, 2008). Biofilm formation in metal piping of industrial settings may result in microbially influenced corrosion (De Carvalho, 2007). Undesirable formation of biofilms on water purification membrane surfaces, known as biofouling, is of particular concern in membrane processes (Flemming et al., 1997), causing notable decline in membrane flux which thus requires higher energy consumption to overcome the biofilm resistance (Komlenic, 2010). While there are many studies reporting detrimental effects of biofilms, numerous studies show the positive aspects of biofilms. For example, biofilm

processes have been used in wastewater treatment for nutrient and contaminant removal (Nicoletta et al., 2000), microbial fuel cells for electricity generation (Min and Logan, 2004), and biocatalysis for chemical production (Halan et al., 2012). The robust nature of biofilms, due to their ability to withstand adverse conditions compared to their planktonic counterparts, makes them promising for many beneficial applications (Flemming and Wingender, 2010; Rosche et al., 2009).

A comprehensive understanding of the fundamentals of biofilm biology is required for us to fully harness the power of beneficial biofilms. Advancement in biofilm research in recent years has enabled designing and improving biofilm bioprocesses for a desirable performance in various chemical, energy and environmental applications. In this review, we provided an overview of biofilm biology focusing on the biofilm life cycle, biofilm matrix and key signaling systems involved in biofilm development and then reviewed recent studies on applying the knowledge and insights to harnessing the power of beneficial biofilms and to combating detrimental biofilms in the context of biotechnology.

2.2 Biofilm biology

2.2.1 Biofilm life cycle

Biofilm formation is a complex, dynamic process comprising of several stages: (1) attachment of cells onto surface/interfaces, which is initially reversible and becomes irreversible; (2) proliferation within the colonized surface and formation of microcolonies; and (3) dispersal (Figure 2.1).

Figure 2.1 The biofilm life cycle. (1) Free swimming bacteria (2) attach to available surfaces. (3) The division of bacteria allows them to stick together and form small microcolonies. (4) With increasing size and density of aggregates, self-produced components are excreted to form the extracellular polymeric substance matrix for maintaining the function and structure of the biofilm. (5) When the biofilm become mature, gradients of factors (e.g., nutrient and oxygen) arise in the biofilm matrix, resulting in the heterogeneous nature of the biofilm. (6) In response to the change of environmental cues, cells “escape” from the matrix. Reproduced with permission from (McDougald et al., 2011) Copyright (2018) Springer Nature.

The attachment of microbial cells onto the surfaces or interfaces is the first step in biofilm formation. Adhesion occurs in two stages, the reversible and irreversible adhesion (Zobell, 1943). Reversible adhesion is generally defined as the initial contact and interaction of microorganisms with a substratum, where they still exhibit Brownian motion and can be easily removed (Rittman, 1982). Initial microbial attachment is mediated by electrostatic and hydrophobic interactions and is generally followed by cell growth and multiplication on the surface. Cell-surface hydrophobic interactions, electrostatic forces

and van der Waals forces contribute to the second phase of biofilm development, referred to as irreversible attachment (Flemming and Wingender, 2010). Irreversible adhesion results in the permanent attachment of bacteria on the surface, mainly facilitated by the self-produced EPS. Bacterial surface appendages such as pili and flagellum provide anchorage and promote the surface attachment of cells (Vigeant et al., 2002).

Surface attachment is followed by biofilm development through the formation of microcolonies by surface-attached microbial cells. Biofilm often comprises of cell clusters, discrete aggregates of cells located within the EPS matrix, which have been shown to vary in size and shape (de Beer et al., 1994). A number of 3-dimensional (3-D) biofilm structures including cylinders, filaments, streamers, stacks and typical mushroom shaped towering structures have been reported (Costerton et al., 1994). The presence of porous channels within microcolonies facilitates the mass transfer of nutrients and water within the biofilm microenvironments (Bishop et al., 1995). EPS keep the microbial cells together in a 3-D scaffold and alter the physico-chemical parameters of the biofilm including the roughness, porosity, hydrophobicity, nutrient gradients and overall biofilm heterogeneity (Flemming et al., 2007; Flemming and Wingender, 2010). Cells in biofilms are usually tightly packed, which aids cell-cell interactions, facilitates metabolites exchange, and promotes synergistic interaction among different organisms (Blenkinsopp and Costerton, 1991). The chemical and physical microenvironments within biofilms are unique features of biofilm structures which contribute to the highly diverse physiology, growth, gene expression and distribution of organisms within these microbial communities (Flemming and Wingender, 2010). As biofilms develop, gradients are formed, including oxygen, pH and nutrient gradients. As a consequence, the bacteria within the biofilms experience varied

physiological conditions which causes the population to be highly heterogeneous (Stewart and Franklin, 2008). Additionally, the EPS impart structural integrity and stability to the biofilm as well as provide resistance to toxic and harsh environments by serving as an external barrier (Flemming et al., 2007; Flemming and Wingender, 2010; Sutherland, 2001). For instance, biofilms are generally highly tolerant to antimicrobial agents and previous studies have shown that bacteria embedded within such structures can be 1,000 times more tolerant to antimicrobials than planktonic cells (Brooun et al., 2000).

The final stage of biofilm development is biofilm dispersal, where microbial cells disseminate from the biofilm into the environment to colonize new niches. Passive detachment generally occurs due to the erosion or sloughing of the biofilms by hydrodynamic sheer forces (Kaplan, 2010; Stoodley, Paul et al., 2001). Active dispersal involving the gradual transition of cells from biofilm mode to planktonic mode can be induced by a variety of environmental cues and processes, including nutrient limitation, oxygen depletion, temperature shift, non-optimal levels of iron, accumulation of waste products, enzyme-mediated breakdown of the biofilm matrix, and overproduction of surfactants (Guilhen et al., 2017; Kaplan, 2010; McDougald et al., 2011). Recent findings have suggested that modulation of the intracellular second messenger c-di-GMP plays an important role in the regulation of biofilm dispersal, high levels of which promote biofilm formation, while low levels induces planktonic mode (Schleheck et al., 2009). Additionally, certain matrix-degrading enzymes such as proteases and cellulases are occasionally released by microorganisms into the matrix that hydrolyze EPS components causing biofilm dispersal (Kaplan et al., 2003).

2.2.2 Biofilm matrix

The slimy consistency of the biofilm is related to the hydrated EPS matrix, occupying approximately 90% of the overall biofilm biovolume (Flemming and Wingender 2010). The biofilm matrix imparts the following functions to biofilms: i) mechanical stability and structural integrity, ii) tolerance against adverse conditions, iii) facilitated diffusion of nutrients, and iv) promoted microbial adhesion by altering the physicochemical characteristics of the colonized surface (Flemming et al., 2007; Flemming and Wingender, 2010). The major components of a biofilm matrix are polysaccharides, proteins and eDNA (Figure 2.2).

Figure 2.2 The biofilm matrix. (A) A model of biofilm formed on a solid surface. (B) The matrix mainly comprises of proteins, DNA and polysaccharides. Adapted with permission from (Flemming and Wingender, 2010) Copyright (2018) Springer Nature.

Extracellular polysaccharides play a pivotal role in various stages of biofilm development (Sutherland, 2001). For example, *Pseudomonas aeruginosa* produces three distinct types of polysaccharides: alginate, Pel and Psl, that contribute towards biofilm formation (Branda et al., 2005; Colvin et al., 2012; Ramsey and Wozniak, 2005). While alginate initiates the formation of microcolonies (Nivens et al., 2001), Pel mediates the formation of air-liquid interface biofilms called pellicles (Friedman and Kolter, 2004; Ghafoor et al., 2011) and Psl facilitates the attachment of cells and maintains the biofilm architecture (Ma et al., 2009). Recent studies have suggested the varied role of matrix associated proteins in biofilm differentiation (Flemming and Wingender, 2010). For instance, the secreted proteins, cellulases and proteases, present in the matrix induced biofilm dispersal by hydrolysing the structural components of EPS (Stoodley et al., 2001; Winn et al., 2012). In addition to the secreted proteins, the structural proteins in the matrix impart stability to the biofilm through interaction with cations such as Ca^{2+} and polysaccharides (Cao et al., 2011a; Cao et al., 2011b; Flemming and Wingender, 2010). Bap, biofilm associated proteins, identified in *Staphylococcus aureus* is an example of high molecular weight structural protein which plays an important role in biofilm formation (Cucarella et al., 2001). Functionally related proteins homologous to Bap have been reported in various phylogenetically diverse bacteria (Lasa and Penadés, 2006). All the Bap homologous proteins exhibit high molecular weight, include a core domain of repeats and

promote bacterial aggregation as well as biofilm formation (Lasa and Penadés, 2006). Furthermore, recent studies demonstrated the role of eDNA in the initial stages of the biofilm life-cycle (Montanaro et al., 2011; Tang et al., 2013). A study in *P. aeruginosa*, has demonstrated that exogenous addition of DNase I in the medium inhibited biofilm formation, whereas medium without DNase I formed extensively colonized biofilms. Additionally, they reported that DNase I treatment on young preformed biofilms (12-36 h old) resulted in biofilm disassembly, whereas DNase I exposure to mature preformed biofilms (84 h old) has minimal effect on biofilm dispersal (Whitchurch et al., 2002). Thus, biofilm matrix provides a dynamic environment for cells in biofilms and plays important roles in biofilm development.

2.2.3 Chemical signaling systems in biofilm development

Bacteria switch their lifestyle between planktonic and sessile modes in response to various environmental cues. This switch is often regulated by sophisticated intracellular signaling networks that modulate the levels of small molecules. Among them, c-di-GMP and quorum sensing signaling networks are the most studied chemical signaling systems.

C-di-GMP is a cyclic dinucleotide consisting of two guanosine monophosphate moieties linked together by phosphodiester bonds and it was first discovered in the bacterium *Gluconacetobacter xylinum* as an allosteric regulator of cellulose synthase (Ross et al., 1990; Ross et al., 1987). C-di-GMP serves as a prevalent intracellular messenger that coordinates the transition between the motile and the sessile lifestyles of many bacterial species (Liang, 2015). Earlier studies have shown that c-di-GMP plays an important role in mediating several cellular functions and various aspects of bacterial physiology (Cotter

and Stibitz, 2007; Hengge, 2009; Tamayo et al., 2007). High levels of c-di-GMP promote biofilm formation by increasing cell aggregation and exopolysaccharide production, whereas low levels of c-di-GMP reduce biofilm formation by increasing bacterial motility and extracellular DNA production (Hu et al., 2017; Wu et al., 2015a). C-di-GMP is synthesized from two molecules of guanosine-5'-triphosphate by diguanylate cyclases (DGCs), while it is degraded into 5'-phosphoguanylyl-(3'-5')-guanosine and guanosine monophosphate by phosphodiesterases (PDEs) (Hengge, 2009). The intracellular concentration of c-di-GMP is controlled by the antagonistic activities of DGCs and PDEs, further regulated by associated sensory domains (Liang, 2015). To trigger cellular responses, c-di-GMP binds to a diverse array of cellular targets or effectors, for example, the PilZ domain (PF07238) present in various proteins (Amikam and Galperin, 2006; Liang, 2015), GGDEF/EAL domains (Qi et al., 2011; Whitney et al., 2012), and riboswitches (Sudarsan et al., 2008). The presence of large number of GGDEF and EAL protein-encoding genes found in diverse bacterial genomes highlights the importance of c-di-GMP mediated signaling in bacteria (Liang, 2015) (Figure 2.3).

Figure 2.3 Structure and physiological functions of c-di-GMP. The cellular level of c-di-GMP is regulated by DGCs with GGDEF domains and specific PDEs with EAL or HD-GYP domains. The c-di-GMP signaling networks coordinate many biological processes, including motility, virulence, biofilm formation and cell cycle. Adapted with permission from (Hengge, 2009) Copyright (2018) Springer Nature.

Pel polysaccharide production in *P. aeruginosa* was induced during biofilm formation (Colvin et al., 2011), which has been reported to be mediated by the local pools of c-di-GMP controlled by several DGCs and PDEs (Liang, 2015). Merritt et al. has previously shown that binding of c-di-GMP to the RoeA and SadC, positively regulated the production of Pel and induced biofilm formation (Merritt et al., 2010). BifA containing an active EAL domain negatively regulated Pel production, while the mutant strain lacking BifA (*ΔbifA*) exhibited an increased c-di-GMP level and induced Pel synthesis (Kuchma et al., 2007). The c-di-GMP responsive transcriptional regulator FleQ in *P. aeruginosa* has

also been demonstrated to repress *pelA* transcription by binding to the *pelA* promoter, whereas a high concentration of c-di-GMP relieved the repression and induced Pel synthesis (Hickman and Harwood, 2008). C-di-GMP has also been shown to bind to the PilZ domain present in various proteins to regulate extracellular polysaccharide synthesis, motility, and flagella activity. For instance, in *Escherichia coli*, binding of c-di-GMP to the PilZ domain of protein YcgR reduced swimming motility and increased biofilm formation (Hengge, 2009). C-di-GMP regulatory mechanisms that regulate EPS production and biofilm formation are substantially diverse as evidenced by the involvement of different c-di-GMP binding effectors and signaling pathway.

Quorum sensing (QS) is cell communication between bacteria, belonging to the same species or to multiple species, to regulate communal behaviors such as bioluminescence, swarming and production of virulence (Eickhoff and Bassler, 2018). Quorum sensing regulation is mediated by extracellular signals that are synthesized and secreted at specific stages of growth. Quorum sensing systems were first discovered in the marine bacterium *Vibrio fischeri* as being associated with the regulation of luciferase gene expression and have been found to be nearly ubiquitous in bacteria, including *Pseudomonas*, *Escherichia*, and *Streptococcus* (Papenfort and Bassler, 2016). Although several quorum-sensing systems are known, based on the types of signals, the two most thoroughly described systems are: (i) the acyl-homoserine lactone systems in many Gram-negative bacteria and (ii) the peptide-based signaling systems in many Gram-positive bacteria. After synthesis, QS signal is exported extracellularly, and eventually its concentration increases proportionally to population density. When the concentration of the signal reaches a threshold, the signal detected by a QS receptor triggers a downstream signal transduction

cascade, eliciting a high cell density-induced gene expression (Hawver et al., 2016) (Figure 2.4).

Figure 2.4 Typical quorum sensing circuits: (A) A Gram-negative one-component and (B) two-component QS system; (C) A Gram-positive one-component and (D) two-component QS system. The figure is prepared based on the studies (Miller, 2001; Waters and Bassler, 2005).

A typical QS module present in Gram-negative bacteria is the *V. fischeri* QS system which contains two proteins, i.e., a synthase LuxI that synthesizes an autoinducer (AI) N-(3-oxohexanoyl)-homoserinelactone and a transcriptional factor LuxR that functions in the recognition of the autoinducer (Waters and Bassler, 2005). Most of AIs are able to passively diffuse across the cell membrane. At low cell density, a low concentration of signal caused by a low amount of LuxI is insufficient to activate LuxR, whereas N-acyl homoserine lactones (AHL)-activated LuxR binds to the promoter P_{lux} to upregulate the transcription of downstream genes when the signal concentration reaches a threshold level at a high cell density. In the most extensively studied model strain *P. aeruginosa* PAO1, there are several QS systems, including LasI/LasR, RhII/RhlR and PQS systems (Pesci et al., 1997). The LasI/LasR and RhII/RhlR systems are the LuxR-type QS systems including the signaling molecules AHLs and the cognate regulators (i.e., LuxR-type proteins). Similar to *V. fischeri*, LasR and RhlR respectively bind to N-(3-oxododecanoyl)-homoserine lactone produced by LasI and N-(butyryl)-homoserine lactone produced by RhII to regulate the expression of target genes. Bacteria use this population effect to regulate phenotypes in a manner that makes them adapt to and survive in continuously changing environments by coupling individual cell responses to population-level alterations.

QS has been shown to play a significant role in biofilm formation (Davies et al., 1998; Labbate et al., 2004; Rice et al., 2005). The influence of QS on biofilm formation was first described in *P. aeruginosa* (Davies et al., 1998). In *P. aeruginosa*, mutations in the *lasI* synthase resulted in the formation of flat and undifferentiated biofilms, in contrast to the highly structured, mature biofilms of the wild type strain (Davies et al., 1998). Another study has shown that both *lasI/R* and *rhII/R* QS systems were involved in eDNA

secretion which was required for the formation of highly structured biofilms (Allesen-Holm et al., 2006). Similarly, in *Pantoea stewartii*, expression of EPS is governed by *esaI/R* QS system which negatively regulated EPS production to enhance bacterial attachment during the early stage of biofilm development (Koutsoudis et al., 2006). The AHL deficient strain (*esaI* mutant) formed undifferentiated thick microbial mats, suggesting that the temporal control of EPS production by QS signaling is essential for the development of highly structured biofilms (Koutsoudis et al., 2006).

In addition to AHL-mediated QS signaling, which is usually species-specific, autoinducer-2 (AI-2)-mediated QS signaling has been described as a universal signal for interspecies communication (Winzer et al., 2002). AI-2 has been shown to increase biofilm formation by enhancing flagella motility (Barrios et al., 2006). Gonzalez et al. has reported that AI-2 dependent QS in *E. coli* stimulates biofilm formation and altered cell motility through enhanced transcription of five motility related genes (Barrios et al., 2006). It is therefore apparent that the highly complex QS regulatory system plays a crucial role in modulating biofilm differentiation for a wide variety of bacteria.

2.3 Biofilm-mediated bioprocess

2.3.1 Biofilms-mediated bioprocess for viroous applications

Extensive studies have demonstrated a wide range of advantages the biofilm mode of life. For example, compared to their planktonic counterparts, biofilms have been reported to be more tolerant to harsh physicochemical conditions. Additionally, biofilms provide long-term retention, stability, high rate of regeneration, and potential to operate as

a continuous system (Gross et al., 2007; Halan et al., 2012). Because of these advantages, biofilms as biocatalysts have shown great promise for applications in industrial and environmental biotechnologies.

There have been numerous studies investigating the applications of biofilms in chemical synthesis, such as *Caldicellulosiruptor saccharolyticus* biofilms for the generation of hydrogen (Groenestijn et al., 2009), *Clostridium beijerinckii* biofilms for the production of butanol (Lienhardt et al., 2002), *Lactobacillus casei* biofilms for the production of lactic acid (Demirci et al., 2003) and recombinant *E. coli* PHL644 biofilms for the biotransformation of haloindole into L-halotryptophan (Tsoligkas, et al., 2011). In addition, the use of electroactive biofilms for microbial electrosynthesis and clean energy generation has attracted considerable attention. These processes produce by-products such as methane, ethanol and other organic compounds (Erable et al., 2010; Rabaey et al., 2007).

Biofilms have become a focus of interest for bioremediation (Singh et al., 2006). For example, biofilms play an important role in remediation of chlorinated aromatic compounds, a group of chemicals that are discharged from chemical industry effluents and widespread in soil and groundwater. They are highly carcinogenic and recalcitrant. Kargey et al. removed 2,4-dichlorophenol (DCP) from synthetic wastewater by DCP-degrading *Pseudomonas putida* (Kargi and Eker, 2005). Lendenmann et al. used a fluidized-bed biofilm reactor containing a mixed culture biofilm for the degradation of a nitroaromatic compounds, an isomeric dinitrotoluene (DNT) (Lendenmann et al., 1998). The use of biofilms for heavy metal removal is another promising application. Heavy metal bioremediation by biofilms can be achieved through several types of microbe-mineral

interactions. Previous studies have shown *Shewanella* can couple bioremediation of organic contaminants with heavy metal reduction (Bretschger et al., 2007; Chaudhuri and Lovley, 2003).

2.3.2 Role of biofilm thickness in biofilm-mediated bioprocesses

In the mediation of bioprocesses by catalytic biofilms, the biofilm thickness plays an important role in affecting biocatalytic activity and mass transformation. Insufficient biofilm biomass results in low biocatalytic activity, whereas excessive biomass growth may pose difficulties for nutrient or substrate diffusion (Gross et al., 2007). Solutes are transferred by diffusion in biofilm systems. Diffusion limitations readily happen because the high cell densities and EPS matrix arrest fluid flow and the diffusion distance is increased by the biofilm growth. When a solute does not react with the biofilm, the reduction of diffusion in biofilm system can be described by the ratio D_e/D_{aq} , where D_e and D_{aq} are the effective diffusion coefficients in the biofilm and in water, respectively (Stewart, 1998, 2003). The ratios of some solutes, termed relative effective diffusivity, have been measured by experiments (Figure 2.5). For instance, there is a 40% reduction in glucose diffusion in biofilms as compared to water (Stewart, 2003).

Figure 2.5 Relative effective diffusion coefficients of selected solutes in biofilms. D_e/D_{aq} is the relative effective diffusion coefficient, where D_e and D_{aq} are the effective diffusion coefficients in the biofilm and in water, respectively. MW represents molecular weight. Adapted from (Stewart, 2003).

The diffusion distance of reacting solute is limited to a certain range. When zero-order kinetics prevail in a homogeneous flat biofilm at steady state, the solute diffusion distance can be expressed as:

$$a = (2D_e S_0 / K_0)^{1/2}$$

Where S_0 is the concentration of substrate at the interface liquid–biofilm and K_0 is the substrate volumetric conversion rate in the biofilms. When the solute is a substrate for microbial growth the volumetric reaction rate is given by $K_0 = \mu X/Y_{xs}$, where μ is the specific growth rate of the microorganism, Y_{xs} is the yield coefficient of biomass on

substrate, and X is the cell density in the biofilm (Stewart, 2003). For example, the penetration depth of oxygen with 6 mg/L concentration is 77 μm in a *P. aeruginosa* biofilm that is assumed to be a flat slab with a cell density of 12 mg/cm³ and growth rate 0.80 h⁻¹. When Y_{xO_2} is 0.85 mg of biomass per mg oxygen. D_{aq} and $D_{\text{e}}/D_{\text{aq}}$ are assumed to be 2.68×10^{-5} cm²/s and 0.57, respectively.

Thus, the biofilm at the base of the flat slab is unproductive when the biofilm thickness is more than maximum penetration distance of the substrate. The productive biofilm catalyst should simultaneously allow for sufficient catalytic biomass and effective solute diffusion. Hence, maintaining the biofilm thickness within an optimal range is of great desire for improving overall performance of biofilm-mediated bioprocesses.

2.4 Biofilm engineering

2.4.1 C-di-GMP-targeted biofilm engineering

A signal cascade mediated intracellular secondary messenger c-di-GMP controls biofilm formation and dispersal in a wide range of bacteria. As mentioned above, decreased concentrations of c-di-GMP reduce biofilm formation, whereas high levels of c-di-GMP promote surface attachment and the formation of biofilms (Hengge, 2009; Jenal et al., 2017). Cellular levels of c-di-GMP are modulated in response to internal and environmental cues, which is achieved by the activities of DGCs and PDEs. Although the complexity of c-di-GMP regulation makes it challenging to precisely control the intracellular c-di-GMP concentration, c-di-GMP can be genetically modulated by the (over)expression of DGCs or PDEs, which could artificially override the endogenous c-di-

GMP signaling network. This strategy has been used in a few studies to obtain an enhanced biofilm-mediated bioprocesses. For example, Wu et al. constructed a *Comamonas testosteroni* strain constitutively expressing YedQ (a c-di-GMP synthase from *E. coli*) and demonstrated enhanced biodegradation of 3-chloroaniline by the engineered *C. testosteroni* biofilm (Wu et al., 2015). This study suggests that manipulating c-di-GMP signaling may be a promising approach to enhance biofilm-based biodegradation of xenobiotic compounds. In a recent study, Benedetti et al. engineered *P. putida* by artificially placing the transcription of *yedQ* under the tight control of a cyclohexanone-responsive expression system. They showed that the presence of cyclohexanone increased the c-di-GMP level of the engineered *P. putida*, resulting in the improved biodegradation of haloalkanes due to the enhanced biofilm formation (Benedetti et al., 2016). Recent studies have also shown that an increased c-di-GMP level can enhance the formation of electroactive biofilms and the performance of bioelectrochemical systems. For example, Liu et al. overexpressed a c-di-GMP biosynthesis gene *ydeH* in *S. oneidensis* and showed enhanced biofilm formation as well as improved bioelectricity generation (Liu et al., 2015).

However, constitutive or chemical-inducible (over)expression of DGCs or PDEs may result in an overly thick or thin biofilm due to the irreversibility of constitutively expressing enzymes as well as limited spatial and temporal resolution of chemicals as inducers. A thin biofilm may provide low activity, while an overly thick biofilm contains unproductive biomass because of the diffusion limitation of solutes (Stewart, 1998, 2003). Therefore, it is desirable to maintain the biofilm thickness within an optimal range that allows for sufficient activity and efficient mass transfer. Hu et al. engineered a NIR light responsive biofilm by expressing a NIR responsive c-di-GMP synthase BphS in *S.*

oneidensis MR-1 and showed that NIR light increased c-di-GMP levels and enhanced biofilm formation on electrodes, subsequently, enhanced bioelectricity generation (Hu et al., 2017). This work demonstrated the feasibility of applying light to “guide” biofilm formation and affect catalytic performance in bioreactors. Since a constantly high DGC activity could result in an undesirably thick biofilm, a blue light activated c-di-GMP hydrolase gene *ebI* could be further introduced to achieve the bi-directional control of c-di-GMP levels (Ryu et al., 2017). Mukherjee et al. (Mukherjee et al., 2018) engineered *E. coli* biofilm whose growth and dispersal can be modulated by light through this dichromatic, optogenetic c-di-GMP gene circuit in which the bacterial cells sense NIR light and blue light to adjust its biofilm formation by regulating the c-di-GMP level. They further demonstrated the potential application of the engineered light-responsive biofilm with quorum quenching activity in mitigating biofouling of water purification FO membranes.

C-di-GMP targeted optogenetic approaches could be widely applicable for designing controllable biofilm-enabled applications. For example, Huang et al. (Huang et al., 2018) engineered *P. aeruginosa* cells as printing materials to form patterned biofilms following prescribed illumination through an optogenetic module which comprises of NIR light responsive BphS and a blue light responsive c-di-GMP hydrolase Blrp1. Such optogenetic manipulation with a high spatial resolution (approximately 10 μm) greatly increases the range of available bioprinting techniques.

Light responsive c-di-GMP enzymes often contain two main domains, i.e., a photoreceptor domain binding with a light-absorbing chromophore and an output domain synthesizing/hydrolyzing c-di-GMP. The presence of light induces a conformational

change of the chromophore-bound photoreceptor domain, which exposes the output domain to alter c-di-GMP levels. Light controls the activities of PDEs and DGCs at a post-translational level in the above studies. By combining a light-sensing module and a transcription factor module, optogenetic tools can be used to control gene expression. Recently, a variety of optogenetic switches, including blue-, green- and red-light responsive systems have been constructed (Fernandez-Rodriguez et al., 2017). Light-switchable transcription modules have been employed in numerous gene circuits for different applications, including metabolic engineering, gene therapy and biofilm engineering (Kennedy et al., 2010; Pu et al., 2018; Zhao et al., 2018). Thus, light responsive biofilms can be achieved by placing biofilm-relevant genes under the control of light-responsive switches. For example, Pu et al. (Pu et al., 2018) engineered a blue light responsive *P. aeruginosa* biofilm by placing a c-di-GMP hydrolase gene under a blue light responsive promoter that is regulated by blue light-responsive transcriptional factors. The dispersal of engineered *P. aeruginosa* biofilm is regulated by blue light through decreasing cellular c-di-GMP levels via the activation of c-di-GMP hydrolase expression.

2.4.2 QS-targeted biofilm engineering

QS is essential for building a biofilm, while disrupting QS can efficiently affect biofilm formation. Previous studies have identified a diverse set of close analogues as quorum sensing inhibitors (QSIs) which exert their effects through binding to the quorum sensing receptors by displacing the cognate AIs (Alhede et al., 2009; Glansdorp et al., 2004). Such an anti-QS approach has been widely applied for combating pathogen bacteria biofilms and identifying drugs (Yang et al., 2009). On the other hand, AHL can be

degraded using four different enzymes, i.e., lactonase, acylase, decarboxylase and deaminase, in a process known as quorum quenching (QQ) (Fetzner, 2015). QQ-based approaches have been used to control biofilm formation on membranes (i.e., biofouling), because QS has been shown to play an essential role in membrane biofouling. Disruption of cell communication by using enzymes to degrade QS signals has been reported to be an effective method to mitigate membrane biofouling in water purification (Gu et al., 2018). For example, Kim et al. showed that using free acylase to degrade AHL reduced biofilm formation by *P. putida* that was isolated from a fouled FO membrane (Paul et al., 2009). With growing interest in the development of synthetic biology for environmental application, QQ gene circuits have been emerged to confer QQ effect on non-QQ bacteria. For example, Mukherjee et al. constructed a QQ *E. coli* strain by introducing a QQ gene circuit comprising a QQ gene (*aiiO*) from *Ochrobactrum anthropi* into *E. coli* (Mukherjee et al., 2018). The biofilm formation by *P. stewartii* (an isolate from wastewater) was significantly reduced on the FO membrane with the pre-formed QQ *E. coli* biofilm compared to the membrane without pre-formed QQ *E. coli* biofilm.

One important aim for synthetic biology is to develop biological systems that perform novel functions by assembling genetic modules (Fu, 2006). To date, synthetic biologists have created a series of genetic modules including switches, cascades, pulse generators, time-delayed circuits, oscillators, spatial patterning and logic formulas (Purnick and Weiss, 2009). These genetic modules achieve the tuning of gene expression, protein production, metabolism and cell–cell communication through controlling transcription, translation and post-translational operations. Since biofilm development is ultimately a genetic process, they can be engineered like other genetic systems using synthetic biology

approaches (Wood et al., 2011). For example, Brenner et al. used the RhII/RhlR QS system to demonstrate roles for self-organization and aggregation in a synthetic biofilm consortium (Brenner et al., 2007).

Hong et al. combined the LasI/LasR QS module of *P. aeruginosa* with engineered biofilm dispersal proteins, Hha and BdcA, and utilized this population-driven QS switch to achieve the control of consortial biofilm formation and dispersal in a microfluidic device (Hong et al., 2012). They also engineered a beneficial *E. coli* strain to inhibit biofilm formation by other bacteria through secreting nitric oxide, a general biofilm dispersal agent (Wood et al., 2016). To control biofilm formation by beneficial bacteria on the membrane, they constructed a QS-based gene circuit to limit its own thickness by sensing the number of its cells via the LasI/LasR QS system of *P. aeruginosa*. The transcription of *bdcA* under the QS promoter P_{lasI} was induced when the autoinducer accumulated. They used QS-driven biofilm dispersal to limit the biofilm quantity and thickness of the protective strain. Taken together, QS system serves as a good target for biofilm control and also provides useful components to construct synthetic networks for biofilm engineering.

2.5 Summary

Biofilm has generally been recognized as an important platform for industrial biotechnological applications such as biocatalysis, energy generation and bioremediation. The fundamentals of biofilm biology can be applied to engineer biofilms for enhanced performance of biofilm-mediated bioprocesses. The biofilm life cycle and diverse regulatory pathways that could be manipulated to engineer biofilms are summarized in Figure 2.6.

Figure 2.6 Schematic illustration of the biofilm life cycle and potential targets for biofilm engineering.

Based on the fundamental studies of biofilm biology, c-di-GMP, and QS have been demonstrated to be promising targets for biofilm engineering. In addition, biofilm biology-informed biofilm engineering can be readily facilitated by the improvement of gene circuit activity through directed protein evolution and the native pathway engineering through gene knockout or knockdown using clustered regularly interspaced short palindromic

repeats (CRISPR)/CRISPR interference (CRISPRi) and Hfq-dependent small regulatory RNA (sRNA). Synthetic biology and genomic editing are powerful tools to improve our understanding of biofilm development. The improved understanding of biofilm biology will further inform biofilm engineering and facilitate practical applications of engineered biofilms.

Chapter 3. **A near-infrared light responsive c-di-GMP module-based AND logic gate in *Shewanella oneidensis***

*This chapter has been published as Yidan Hu, Yichao Wu, Manisha Mukherjee and Bin Cao (2017) A near-infrared light responsive c-di-GMP module-based AND logic gate in Shewanella oneidensis. **Chemical Communications** 10: 1646 -1648. Permission has been granted by the licensed content publisher “Royal Society of Chemistry” to use the published content as a chapter in this thesis.*

3.1 Introduction

Microbial fuel cell (MFC), a bioelectrochemical device that generates an electrical current through electron transfer between microorganisms and electrodes, has attracted extensive attention in the recent decades (Rabaey and Verstraete, 2005). With the rapid development in electromicrobiology, MFCs have been demonstrated to be promising in various applications ranging from energy efficient wastewater treatment (Du et al., 2007) to self-powered sensors (Li et al., 2011). Recently, MFCs have been employed in information-processing systems for biocomputing. For example, logic gate-controlled MFCs have been developed to respond to chemicals, such as isopropyl β -D-thiogalactoside (IPTG) (Hu et al., 2015) and quorum sensing signals (Hu et al., 2015; Li et al., 2011), through the activation of the promoters controlling the expression of genes involved in the extracellular electron transfer (EET), though the EET manipulation was conducted at the

individual cell level. Compared to planktonic individual cells, sessile populations or communities on the electrodes, *i.e.*, electrode biofilms, often play a more important role in determining the overall EET performance in MFCs (Borole et al., 2011). However, to the best of our knowledge, there has been no literature reporting biofilm-based logic control in MFCs, mostly because controlling biofilms is very challenging due to the highly dynamic nature of a biofilm.

Herein, we developed a novel, biofilm-based logic control strategy in a MFC by adjusting the thickness of an electrode biofilm through mediating c-di-GMP signaling. The c-di-GMP is one important signaling molecule in biofilm regulatory networks that coordinate biofilm formation and detachment in a wide range of bacteria. Previous studies have shown that an increased c-di-GMP level facilitates biofilm formation, whereas a decrease drives a switch from biofilm to planktonic phenotypes (Hengge, 2009). On cellular level, the c-di-GMP level is mediated by two groups of enzymes, *i.e.*, DGCs which catalyze the synthesis of c-di-GMP, and PDEs which degrade c-di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) or two GMP (Hengge, 2009). Hence, it provides a potential genetic manipulation for logic control of MFCs by adjusting biofilm formation. Moreover, since many c-di-GMP enzymes contain domains capable of sensing physical inputs, such as light (Tarutina et al., 2006), the input range for the logic control can be further broadened.

In this study, we constructed a synthetic NIR light responsive c-di-GMP module through synthetic biology approaches. The NIR light responsive c-di-GMP module comprises an engineered protein BphS (a light-activated diguanylate cyclase) (Ryu and

Gomelsky, 2014) and BphO (a chromophore biliverdin (BV) synthase). The expression of BphS is controlled by IPTG-inducible promoter *P_{tac}*. The conformational change of BV-bound BphS arises under NIR light, which results in sequential changes including the activation of its DGC activity, increased c-di-GMP level, and enhanced biofilm formation. The expression and activation of BphO are respectively controlled by IPTG and NIR light. The expressed BphS has DGC activity only when IPTG and NIR light are present simultaneously, which accomplishes AND logic gate gene circuits. we introduced this synthetic NIR light responsive c-di-GMP module into *Shewanella oneidensis* MR-1. As a result of enhanced biofilm formation on the electrode, the maximum power density (MPD) with input (NIR light, IPTG) was ~2-fold of the MPDs for other input combinations, *i.e.*, no inputs, only NIR light, only IPTG.

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. Stock cultures of *S. oneidensis* MR-1 and *Escherichia coli* strains were stored in Lysogeny broth (LB) medium supplemented with 25% glycerol at -80 °C. Overnight cultures of *S. oneidensis* MR-1 or *E. coli* were grown in LB medium at 30 °C and 37 °C, respectively. The culture medium and buffer solution preparation are shown in Table 3.2. Whenever needed, 50 µg/mL kanamycin was added to the culture medium. The plasmid transformation of *S. oneidensis* needs a helper strain, *i.e.*, *E. coli* WM3064 (a *dap* auxotroph, derivative of strain B2155). 0.3 mM diaminopimelic acid (DAP) was added to the medium when growing *E. coli* WM3064.

Table 3.1 Bacterial strains and plasmids used in this study

Bacterial strains	Relevant characteristics	Reference
<i>E. coli</i>		
DH5 α	The strain for gene clone	(Miller and Nickoloff, 1995)
WM3064	The donor strain for conjugation; a <i>dap</i> auxotroph, derivative of strain B2155	(Dehio and Meyer, 1997)
<i>S. oneidensis</i> MR-1	Environmental and electroactive strain	(Myers and Nealson, 1988)
Plasmids		
pUC57	Amp ^R ; <i>oriV</i> (ColE1), <i>bphS</i> , <i>bphO</i>	This study
pYYDT	Km ^R ; <i>oriV</i> (pBBR1), <i>Ptac</i>	(Yang et al., 2015)
pYYDT-L	Km ^R ; <i>oriV</i> (pBBR1), <i>Ptac</i> , <i>bphS</i> , <i>bphO</i>	This study

Abbreviations used in this table are as follows: Amp, ampicillin; Km, kanamycin.

Table 3.2 Culture medium and buffer solution preparation.

LB medium		Modified M9 medium		Phosphate buffer solution	
NaCl	5 g/L	Na ₂ HPO ₄	6 g /L	NaCl	8.0 g/L
Tryptone	10 g/L	KH ₂ PO ₄	3 g/L	Na ₂ HPO ₄ ·12H ₂ O	3.23 g/L
Yeast extract	5 g/L	NaCl	0.5 g/L	NaH ₂ PO ₄ ·2H ₂ O	0.45 g/L
		NH ₄ Cl	1 g/L	pH 7.0	
		MgSO ₄	1 mM		

3.2.2 Gene circuit construction and transformation

To accelerate gene circuit construction, the gene parts used in this study were designed as BioBricks parts that are compatible with standardized gene expression tools in *S. oneidensis* (Yang et al., 2015). BioBricks, standardized molecular building blocks, consist of BioBrick parts (e.g., promoters, ribosome binding sites (RBSs), coding sequences) and backbones (Figure 3.1A).

Figure 3.1 The illustration for BioBrick (A) and the procedure of PYYDT-L construction (B). (A) The structure of a BioBrick. It consists of a BioBrick part and a plasmid backbone.

BioBrick parts are functional DNA such as promoters, RBSs, Coding sequences, or terminators. BioBrick parts are flanked by an upstream prefix (including EcoRI and XbaI) and a downstream suffix (including SpeI and PstI) for convenient and fast assembly. (B) The procedure of PYYDT-L construction. IPTG inducible promoter *Ptac* and double terminator (shown by TT in plasmid map) were included in plasmid pYYDT. The gene *bphS* and *bphO* as an operon was synthesized *in vitro* and designed as a BioBrick part. Promoter *Ptac* to be placed in the front was cut by SpeI and SbfI, and *bphS-bphO* operon to be placed in the back was cut by XbaI and PstI. The two cut fragments were ligated and formed a long fragment containing *Ptac* and the synthetic operon still in BioBrick format, as the sticky ends of XbaI and SpeI are compatible. Due to PstI cutting site in pYYDT, SbfI was utilized to replace PstI as their sticky ends are compatible. The *bphS-bphO* operon was inserted into pYYDT, which are named as pYYDT-L. PYYDT-L contains three key elements, IPTG inducible promoter *Ptac*, diguanylate cyclase gene *bphS* and heme oxygenase gene *bphO*. Blue circles represent the RBSs. Sufficient translation of key components in the gene circuit was ensured by the strong RBS1 in front of *bphS* and *bphO*.

BioBrick part is flanked by an upstream prefix (including EcoRI and XbaI) and a downstream suffix (with SpeI and PstI). The upstream BioBrick part is digested with SpeI and PstI, while the downstream BioBrick part is digested by XbaI and PstI. PstI was replaced by SbfI when PstI is present in the vector, as the sticky ends of SbfI and PstI are compatible. The two fragments can be ligated together to form the resulting BioBrick, as the sticky ends of XbaI and SpeI are compatible (Figure 3.1A). The key feature of the BioBrick is that it can maintain its format (*i.e.*, BioBrick part flanked by an upstream prefix

and a downstream suffix, and a backbone) after multiple gene part assembly, which facilitates convenient and fast assembly of genetic circuits.

The light responsive synthetic c-di-GMP module is composed of two genes, *bphS* and *bphO*. The sequence of *bphS* was obtained by reverse translation of the protein sequence of engineered BphS (Ryu and Gomelsky, 2014). The sequence of *bphO* (RSP_4190) originating from *Rhodobacter sphaeroides* was extracted from database KEGG (Kyoto Encyclopedia of Genes and Genomes). To ensure optimal translation, the sequences of *bphS* and *bphO* were optimized in *S. oneidensis* using JCAT (Java Codon Adaptation Tool), and restriction enzyme sites of EcoRI, XbaI, SpeI, and PstI were avoided in optimized sequences. RBS-*bphS*-RBS-*bphO* operon flanked by an upstream prefix (E/X) and a downstream suffix (S/P) was synthesized *in vitro* and inserted into the vector pUC57 (GenScript, Singapore). The sequence of the synthetic operon was confirmed by primers M13-F and M13-R. To insert the synthetic operon into the vector available in *S. oneidensis* (*i.e.*, pYYDT), the *bphS-bphO* operon was amplified by polymerase chain reaction (PCR) (primers Biobrick-F and Biobrick-R), and then digested by restriction enzymes (XbaI and PstI). Sequentially, the fragment of *bphS-bphO* was directly ligated with pYYDT digested by SpeI and SbfI to form pYYDT-L (Figure 3.1B). The ligation product was transferred into *E. coli* DH5 α competent cells. To select colonies with pYYDT-L, colony PCR was conducted using primers YYD-F and YYD-R, and the constructs were sequenced (AIT biotech, Singapore). The primers and gene circuit sequence are listed in Table 3.3 and Table 3.4, respectively.

Table 3.3 The primers used in this study

Primer name	Primer sequence	Reactions
M13-F	GTTTTCCCAGTCACGAC	Sequence primers of pUC57
M13-R	AACAGCTATGACCATG	
Biobrick-F	GCAGAATTC GCGGCCGC T TCTAGA	<i>bphS-bphO</i> operon amplification
Biobrick-R	ATACCTGCAGGCGGCCGCTACTAGTAT	
YYD-F	TATCAGGCTCTGGGAGGCAG	Sequence primers of pYYDT
YYD-R	TTCTGGACCAGTTGCGTGAGCG	
S-1	CGTGGACCGCTTGCTGCAACT	Sequence primers of pYYDT-L
S-2	CACTACCCAGCTTCTGATATCC	
S-3	CGTGCTTCTTTCGATGCTTG	

Table 3.4 Sequence of NIR light responsive c-di-GMP module

EcoRI-XbaI-RBS1-<i>bphS</i>-RBS1-<i>bphO</i>-SpeI-SbfI (2739bp)
<p>EcoRI-XbaI (GCAGAATTCGCGGCCGCTTCTAGAG)</p> <p>RBS1 (aaggagatatacat) <i>bphS</i> (ATGGCTCGTGTTGTTTAATGACTATCTCTGGTGGTACTTTTCGATCCATCTATCTGTGA AATGGAACCAATCGCTACTCCAGGTGCTATCCAACCACACGGTGCTTTAATGACTGCTCGTGCTGATTCTGGTCGTGTTGCTCA CGCTTCTGTAACTTAGGTGAAATCTTAGGTTTACCAGCTGCTTCTGTTTAGGTGCTCCAATCGGTGAAGTTATCGGTCGTGT TAACGAAATCTTATTACGTGAAGCTCGTCGTTCTGTTCTGAAACTCCAGAACTATCGGTTCTTCCGTCGTTCTGATGGTCA ATTATTACACTTACACGCTTTCCAATCTGGTGATTACATGTGTTAGATATCGAACCAGTTCGTGATGAAGATGGTCGTTTACC ACCAGGTGCTCGTCAATCTGTTATCGAACTTTCTCTTCTGCTATGACTCAAGTTGAATTATGTGAGTTAGCGGTTACAGGTCT CCAATTAGTTCTCGGTTACGATCGTGTTATGGCTTACCGTTTCGGTGCTGATGGTCACGGTGAAGTTATCGCTGAACGTCGTCG TCAAGATTTAGAACCATACTTAGGTTTACACTACCCAGCTTCTGATATCCACAAATCGCTCGTGCTTTATACTTACGTCAACG TGTGGTGCTATCGCTGATGCTTGTACCGTCCAGTTCATTATTAGGTCACCCAGAATTAGATGATGGTAAACCATTAGATTT AACTCACTCTTCTTACGTTCTGTTTCTCCAGTTCAGTTAGATTACATGCAAAACATGAACACTGCTGCTTCTTAACTATCGG TTTAGCTGATGGTGATCGTTTATGGGTATGTTAGTTTGTCAACACTACTCCACGTATCGCTGGTCCAGAATGGCGTGCTGC TGCTGGTATGATCGGTCAAGTTGTTTCTTTATTATTATCTCGTTAGGTGAAGTTGAAAACGCTGCTGAACTTTAGCTCGTCA ATCTACTTTATCTACTTTAGTTGAACGTTTATCTACTGGTGATACTTTAGCTGCTGCTTTCGTTGCTGCTGATCAATTAATCTT AGATTTAGTTGGTGCTTCTGCTGCTGTTGTTTCGTTTAGCTGGTCAAGAATTACACTTCGGTCGTAAGTTCACCTGATGCTAT GCAAAAAGTTTATAGATTCTTTAGGTCGTCATCTCCATTAGAAGTTTATCTTTAGATGATGTTACTTTACGTCACCCAGAATT ACCAGAATTATTAGCTGCTGGTCTGGTATCTTATTATTACCATTAACCTTCTGGTGACGGCGATCTCATAGCGTGGTTCGCCCC AGAACACGTTCAAATATCACTTGGGGTGGTAACCCAGCTGAACACGGTACTTGAACCCAGCTACTCAACGTATGCGTCCACG TGCTTCTTTCGATGCTTGGAAAGAACTGTTACTGGTCGTTCTTACCATGGACTTCTGCTGAACGTAAGTGTGCTCGTGAATT AGGTGAAGCTATCGCTGCTGAAATGGCTCAACGTAAGTCTGCTGAAAGTGAACGTTGCTATGGTTGATTCTTTAACTCG TTTATGGAACCGTTTAGGTATCGAACTTTATTTAAACGTTGAATGGGAATACGCTACTCGTAAAACTCTCAATCTCTATCGT TATGATCGATTTTCGATAACTCAAACAAATCAACGATCAACACGGTCACTTAGTTGGTGATGAAGTTTACAAGTTCTGCTCG</p>

3.2.4 Confocal laser scanning microscopy (CLSM) and image analysis

To quantify the biovolume, the same biofilms grown on the bottom of the wells were imaged using confocal scanning microscopy (CLSM, Carl Zeiss Microscopy LSM 780). After removing planktonic cells, the cells were stained by 2.5 μ M SYTO 9 (a green fluorescent nucleic acid stain). All experiments were conducted with three biological replicates and three randomly picked images were taken for each replicate. Biofilm biovolume was calculated using IMARIS (version 7.6.4, Bitplane, Zurich, Switzerland).

3.2.5 MFC set up

The previous method was used (Hu et al., 2015). Two-chamber MFCs separated by Nafion 117 (Gashub, Singapore) were used in the experiment. The Nafion 117 membrane was pretreated as follows. First, it was boiled in 3% hydrogen peroxide for 30 min at 80 °C in a water bath. After being washed with distilled water several times, it was then boiled in distilled water for 30 min at 80 °C. Finally, it was boiled in 0.5 M sulfuric acid for 30 min at 80 °C, washed, and kept in sterile distilled water before MFC setup. Carbon cloth (Gashub, Singapore) was used as the electrodes for both anode (2.5 cm $\times$ 2.5 cm, *i.e.*, the geometric area is 6.25 cm²) and cathode (2.5 cm $\times$ 3 cm). The catholyte consists of 50 mM K₃[Fe(CN)₆], 50 mM K₂HPO₄ and KH₂PO₄. The anode chamber was filled with 150 mL of optimal medium. This optimal medium was M9 medium supplemented with 5% LB and 20 mM lactate. One mL of overnight culture was transferred into 100 mL fresh LB medium with or without the induction of 0.5 mM IPTG, respectively. After 8 h incubation (30 °C, 200 rpm shaking), the cells were harvested and washed three times with PBS and then injected into anode chamber at an optical density (OD_{600nm}) of 0.4. After inoculation, each

MFC electrolyte was bubbled with nitrogen for 3 mins. A 1 k Ω external resistor was connected to both anode and cathode. MFC reactors were incubated at 30 °C under four conditions (*i.e.*, no IPTG or NIR light, only IPTG (0.5 mM), only NIR light (660 nm, 7.02 mW/cm²), and IPTG (0.5 mM) and NIR light (660 nm, 7.02 mW/cm²)), and the voltage outputs were recorded.

3.2.6 The measurement of MFC power density

To investigate the performance of the MFCs further, the polarization curves were measured by recording the voltage output at various external resistances (200k–1k Ω) when the electrical outputs of MFCs were the highest (Liu et al., 2015) (approximately 48 h incubation). The voltage output was recorded after connecting the resistor when the output was stable. The current density and power density were calculated respectively by relation $I=V/R \cdot S$ and $P=VI$, where V, R and S represent voltage (V), resistance (R) and carbon cloth area (S). From polarization curves, the maximum power densities with four input combinations were obtained.

3.2.7 Electrode-attached biofilm images and biomass measurement

To verify that our gene circuit can control biofilm formation under anaerobic conditions, the confocal images of biofilms on anodes were taken. The planktonic cells on electrodes were removed by washing with PBS. Subsequently, the carbon cloths were stained by SYTO 9. After staining for 15 min in the dark, the biofilms on electrode were imaged using CLSM. Attached biomass on electrodes was further measured. The carbon cloths were cut into small pieces and immersed in 0.1 mM NaOH to lyse cells by incubating

at 70 °C for 1 h. Total protein concentrations were measured by Qubit protein assay kit (Invitrogen) and a Qubit fluorometer.

3.3 Results and discussion

3.3.1 Design of the genetic circuits

The NIR light responsive c-di-GMP module comprises an engineered protein BphS (a bacteriophytochrome c-di-GMP synthase) (Ryu and Gomelsky, 2014) and BphO (a chromophore BV synthase from *Rhodobacter sphaeroides*) (Figure 3.2A). BphS contains two main domains, *i.e.*, a typical photoreceptor module (PAS-GAF-PHY domain) which binds to the light-absorbing chromophore (BV in our design), and a typical DGC output domain (GGDEF domain) which can synthesize intracellular c-di-GMP to enhance biofilm formation. Under NIR light, conformational change of BV-bound PAS-GAF-PHY domain is efficiently transduced to the GGDEF domain, leading to the activation of its DGC activity. Gene *bphO* is located immediately downstream of gene *bphS* to form an artificial *bphS-bphO* operon (Figure 3.2A). The *bphS-bphO* operon is directly controlled by IPTG inducible promoter, *Ptac*. Therefore, when IPTG and NIR light are present simultaneously, the expressed BphS has DGC activity, which results in an increased c-di-GMP level and subsequently enhanced biofilm formation on anodes to increase electrical output (Figure 3.2B). Since previous study showed a very low level (~50 µM) of ferricyanide can diffuse across the proton exchange membrane to enter the anode chamber from the cathode chamber (Yong et al., 2012), its effect on the electrochemical test is not considered in this system.

Figure 3.2 (A) Schematic illustration of NIR light responsive c-di-GMP module based-AND logic gate in MFC. (B) Truth table of the AND logic gate.

3.3.2 Verification of the genetic circuits by static biofilm assay

As the IPTG-inducible promoter allows various expression levels of the NIR light responsive c-di-GMP module upon differential IPTG induction, we optimized IPTG concentration by the conduction of static biofilm assays using 24-well microtiter plates. The recombinant *S. oneidensis* cultures were incubated under NIR light (660 nm, 7.02 mW/cm²) and at different IPTG induction levels. Biofilm biovolume was obtained using confocal images analysis and is shown in Figure 3.3A. The biovolume of biofilm induced by 0.5 mM IPTG was significantly higher than that induced by 0.1 mM IPTG ($P < 0.001$),

while it was comparable to that induced by 1 mM IPTG ($P > 0.1$). It indicates that 0.5 mM IPTG was sufficient for a saturated expression level of the NIR light responsive c-di-GMP module. In addition, we tested the static biofilm formation of recombinant *S. oneidensis* strain using two different light intensities (7.06 mW/cm² and 15.80 mW/cm²). The results showed that an increase in the NIR light intensity did not further augment biofilm formation, probably reflecting a saturation of the system at 7.06 mW/cm² (Figure 3.3B).

Figure 3.3 The performance of NIR light responsive c-di-GMP module for biofilm formation upon the induction of IPTG with different concentrations (A) and NIR light with different intensities (B). *** $P < 0.0005$, Student's t test.

To test the biofilm-based NIR light responsive AND logic circuit, we conducted static biofilm assays. Cultures were incubated under the following conditions: (i) no IPTG or NIR light, denoted as (0, 0); (ii) only IPTG (0.5 mM) or (1, 0); (iii) only NIR light (660 nm, 7.02 mW/cm²) or (0, 1); and (iv) IPTG (0.5 mM) and NIR light (660 nm, 7.02 mW/cm²) or (1, 1). CLSM images of biofilms and the amount of biofilm biomass under different conditions after 24 h are shown in Figure 3.4.

Figure 3.4 The performance of AND logic gate gene circuit for biofilm formation. (A) CLSM images of the static biofilm of *S. oneidensis* harbouring the constructed gene circuit and (B) the empty vector. (C) Biofilm biovolume per unit area of the bottom of each well and (D) total biofilm biomass (OD_{550nm}) in each well. *S. oneidensis* harboring the

constructed gene circuit and the empty vector (E, F) was incubated for 24 h under four input conditions: (0, 0), no IPTG or NIR light; (1, 0), only IPTG; (0, 1), only NIR light; and (1, 1), IPTG and NIR light. Experiments were conducted in triplicates. The scale bar represents 20 μm . * $P < 0.05$, ** $P < 0.005$, Student's t test.

The biofilm biomass was obtained using crystal violet assay. CLSM images of the static biofilm of *S. oneidensis* harbouring the constructed gene circuit are shown in Figure 3.4A. As IPTG-induced BphS has DGC activity only under the NIR light condition, biovolume and the amount of biofilm biomass indicated by OD_{550nm} were drastically higher (~3-fold for biovolume and ~7-fold for biofilm biomass) when the input was (1,1) as compared to the other three input combinations where the biofilm formation capability was low. CLSM images of the static biofilm of *S. oneidensis* harbouring the vector are shown in Figure 3.4B. The increases in biovolume and biofilm biomass were not observed in the static biofilm assay of the control strain, *i.e.*, *S. oneidensis* MR-1 harboring the empty vector pYYDT (Figure 3.4E and F). In addition, under our tested conditions, the intracellular c-di-GMP level of the *S. oneidensis* cells expressing *bphS*–*bphO* with NIR light was more than 5-fold that without NIR light (5.76 vs. 1.02 pmol μg^{-1} total protein). These results showed a reasonably low basal background in the host cells and the effectiveness of the biofilm-based AND logic gate.

3.3.3 Biofilm-based AND logic in MFC

We then further demonstrated the biofilm-based AND logic control in MFCs. The MFCs inoculated with the strain harbouring the AND-gated gene circuit were incubated at

30 °C under dark or NIR light to achieve four different inputs: no inputs (0, 0); only IPTG (1, 0); only NIR light (0, 1); and both IPTG and NIR light (1, 1). The output voltage (V) was recorded across a 1 k Ω resistor with three biological replicates for each condition. To observe biofilm formation on the electrode, confocal images of the biofilms formed on the anodes were obtained using CLSM, when the voltage output reached the maximum value (Figure 3.5A).

Figure 3.5 The performance of AND logic gate gene circuit for biofilm formation on the electrodes in MFC system. (A) CLSM images of biofilms and (B) The attached biomass (in terms of total proteins) of *S. oneidensis* harboring the constructed gene circuit on the anodes of AND logic gate-controlled MFCs. MFCs experiments were conducted in triplicates for each condition. Images were taken at three random spots on each electrode, and the representative image is shown here. The scale bar represents 50 μm . **P < 0.005, Student's *t* test.

More biofilm biomass was found on the electrode in MFCs operated in the presence of both IPTG and NIR light, i.e., input (1, 1) than those operated in the conditions with inputs (0, 0), (0, 1) or (1, 0). To further quantify the biofilms on the electrodes, total protein concentration was measured. The biofilm biomass with the input (1, 1) was ~ 4 -fold of those under input conditions (1, 0), (0, 1) and (0, 0) (Figure 3.5B).

To further examine whether the logic gated-biofilm formation on electrodes is correlated with the electric signal output in MFCs, the polarization curves were obtained by recording the voltage output at various external resistances (200 k Ω - 1 k Ω) (Figure 3.6A).

Figure 3.6 The performance of the AND logic gate gene circuit in MFCs. (A) and (D) Polarization curves, (B) and (E) power density curves, and (C) and (F) maximum output power densities of the MFCs inoculated with the strain harbouring the AND-gated gene circuit or the control strain, respectively, under conditions with different inputs: (0, 0), no

IPTG or NIR light; (1, 0), only IPTG; (0, 1), only NIR light; and (1, 1), IPTG and NIR light. The polarization curves were measured by recording the voltage output at various external resistances (200k–1k Ω). The current density and power density were calculated respectively using the formulae $I=V/(R \cdot S)$ and $P=VI$, where V, R and S represent voltage (V), resistance (R) and carbon cloth area (S) respectively. *** $P < 0.0005$, Student's t test.

From the polarization curves, we obtained the maximum power densities with four input combinations, *i.e.*, 9.94 ± 0.67 mW/m² (no input), 11.45 ± 0.66 mW/m² (only NIR light), 11.38 ± 0.59 mW/m² (only IPTG) and 23.01 ± 1.09 mW/m² (IPTG and NIR light) (Figure 3.6C). The MPD with input (1, 1) was ~2-fold of the MPDs for other input combinations, which may be attributed to the enhanced biofilm formation on the electrodes. In MFC systems, enhanced biofilm formation on anodes increases electrical output in a certain range, which could be because several gradients, including substrate concentration, local potential and proton accumulation, in electroactive biofilms may affect power production (Franks et al., 2014). In addition, the enhanced biofilm matrix could potentially resist electron transfer (Borole et al., 2011). As a result, 6 -fold increase in biofilm biomass on the anode only contributed 2-fold increase in MPDs. Based on different MPDs, the output of the AND logic gate was defined as “0” (OFF) when the maximum power density of MFCs was below the threshold and “1” (ON) when maximum power density of MFCs was above the threshold (threshold value: 13 mW/m²). In addition, the MFCs were inoculated with the control strain. The MPDs of four inputs were 11.83 ± 0.62 mW/m² (no input), 11.56 ± 0.32 mW/m² (only NIR light), 11.89 ± 0.65 mW/m² (only IPTG) and 9.57 ± 0.78 mW/m² (IPTG and NIR light), respectively (Figure 3.6F). There was no significant

difference of MPDs under the four different conditions, suggesting that the environmental or operational conditions have no significant effects on the outputs of the logic gate constructed in this study.

3.4 Conclusion

In summary, we constructed an AND logic gate gene circuit capable of logically regulating biofilm formation and demonstrated the NIR light-responsive, biofilm-based AND logic gated-MFCs. Multiple-input genetic logic gates are of great help for cell to improve the sensing specificity, increase the control accuracy, and stabilize the inactivated off state. These advantages can greatly improve the performance of MFCs as bacteria-based biosensor. In addition, to the best of our knowledge, this is the first report on light-responsive synthetic c-di-GMP module for biofilm-based logic control of MFCs. Since c-di-GMP is a universal biofilm regulator, the approaches reported here will likely work for other exoelectrogens too. Moreover, because of the non-invasibility, controllability and penetrability of NIR light, our gene circuit can potentially be used to construct NIR light-controllable biofilms for other applications, such as bioremediation and biochemical production.

Chapter 4. **Optogenetic modulation of a catalytic biofilm for biotransformation of indole into tryptophan**

*This chapter has been published as Yidan Hu, Xiaobo Liu, Aloysius Teng Min Ren, Ji-Dong Gu, Bin Cao (2019) Optogenetic modulation of a catalytic biofilm for biotransformation of indole into tryptophan. **ChemSusChem** 12: 1-8. Permission has been granted by the licensed content publisher “John Wiley and Sons” to use the published content as a chapter in this thesis.*

4.1 Introduction

Although there is an increasing trend of using biocatalysts (e.g., enzymes and whole cells) in the production of fine chemicals (Jiang et al., 2018; Peschke et al., 2017; Zhao, 2011), there are still some major challenges, including the sensitivity of the biocatalysts to harsh conditions and the reusability of the biocatalysts in continuous bioprocesses (Demirjian et al., 2001). Interface/surface-associated multicellular microbial aggregates or biofilms have been demonstrated in recent studies to be promising biocatalysts for chemical synthesis because of their natural immobilization and high tolerance against physicochemical stresses (Gross et al., 2013; Gross et al., 2010; Halan et al., 2012; Rosche et al., 2009). The robustness of biofilms, which is conferred by a well-organized 3-dimensional architecture where cells are embedded within a self-produced extracellular matrix, allows them to maintain a long-term stability in biofilm-mediated bioprocesses (Flemming and Wingender, 2010; Harrison et al., 2007; Henriques and Love, 2007).

Biofilm-mediated bioprocesses could be improved by enhancing biofilm formation (Ding et al., 2014; Liu et al., 2015; Perni et al., 2013; Wu et al., 2015). In a wide range of bacteria, the development of biofilm is subject to a signal cascade regulated by the intracellular levels of secondary messenger c-di-GMP (Cotter and Stibitz, 2007; Hengge, 2009; Schirmer and Jenal, 2009). Typically, an increase in c-di-GMP level enhances biofilm formation while a decrease in c-di-GMP level leads to biofilm dispersal (Römling et al., 2005). Intracellular levels of c-di-GMP are modulated in response to internal and environmental cues, which is achieved by the activities of DGCs (c-di-GMP synthases) and PDEs (c-di-GMP hydrolases) (Simm et al., 2004). Hence, biofilm formation can be modulated by controlling the expression of genes encoding DGCs or PDEs using chemical inducers. This strategy has been used in a few studies to obtain an enhanced biofilm-mediated bioprocess. For example, overexpression of a c-di-GMP synthase gene *yedQ* in *Comamonas testosteroni* enhanced biofilm formation and improved biodegradation of an industrial pollutant (Wu et al., 2015). Similarly, overexpression of *ydeH* in *Shewanella oneidensis* resulted in thicker biofilms on electrodes and increased bioelectricity generation in microbial fuel cells (Liu et al., 2015). A recent study engineered *Pseudomonas putida* by artificially placing the transcription of a c-di-GMP synthase gene *yedQ* under the tight control of a cyclohexanone-responsive expression system, leading to an enhanced biofilm formation and improved biodegradation of haloalkanes (Benedetti et al., 2016).

For biofilm-mediated biocatalysis for chemicals synthesis, it would be desirable if chemical inducers could be avoided for modulating biofilm formation. Light as a non-invasive stimulus is such an attractive inducer, which can offer a higher spatiotemporal resolution in the regulation of biological activities (Fenno et al., 2011). Several light-

responsive regulatory modules have been reported for protein degradation (Renicke et al., 2013), protein–protein interactions (Levskaya et al., 2009; Yazawa et al., 2009) and gene expression (Levskaya et al., 2005; Müller and Weber, 2013; Ohlendorf et al., 2012). In particular, the NIR light (680–880 nm) (Weissleder, 2001) is an attractive inducer for the photocontrol of biological activities because of its deep penetration and non-toxicity (Petrov et al., 2012; Piatkevich et al., 2013).

In this work, we engineered a NIR light-responsive, tryptophan-producing *Escherichia coli* biofilm by introducing a NIR light-responsive DGC (Ryu and Gomelsky, 2014) and the genes encoding tryptophan synthase (TrpAB) (Kawasaki et al., 1987). Biofilm formation of the engineered *E. coli* could be modulated by NIR light through manipulation of the intracellular c-di-GMP concentration and the biotransformation of indole and serine into tryptophan was catalyzed by TrpAB. The engineered catalytic biofilm overcame the inhibition of indole on biofilm formation by *E. coli* and reduced the reverse reaction from tryptophan to indole.

4.2 Materials and methods

4.2.1 Plasmids, bacterial strains and growth conditions

The pSTB7, a pBR322-based plasmid containing *trpA* and *trpB* genes (from *Salmonella enterica*) with Amp resistance (Kawasaki et al., 1987), was obtained from the American Type Culture Collection (ATCC 37845). The pYYDT-L, a pBBR1-based plasmid containing a NIR light responsive c-di-GMP gene circuit with Km resistance, was constructed based on the empty vector pYYDT (Yang et al., 2015) used in our previous

work (Hu et al., 2017). Two plasmids, pSTB7 with pYYDT (an empty vector) or pSTB7 with pYYDT-L, were transformed into *E. coli* BL21(DE3) using the heat-shock method. Transformants were selected on LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) supplemented with 100 µg/mL of Amp and 50 µg/mL of Km. The engineered *E. coli* BL21(DE3) was pre-cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) at 37 °C. When needed, 100 µg/mL of Amp and 50 µg/mL of Km were added in the culture media.

4.2.2 Congo red assay

The colony morphology was assayed on Congo red agar plate with LB agar containing 50 µg/mL of Km, 100 µg/mL of Amp and 50 µg/mL of Congo red (Sigma-Aldrich) (Fang and Gomelsky, 2010). Strains BL21/ (pSTB7+pYYDT) and BL21/ (pSTB7+pYYDT-L) were grown in LB media at 37 °C for 16 h. Five µl of each overnight culture ($OD_{600nm} \sim 1.2$) was spotted on the Congo red agar plate then incubated for 36 h at room temperature (25-28 °C) under the NIR light (660 nm, 1.33 mW/cm²) or in the dark .

4.2.3 Biofilm formation assay

Biofilm assay was conducted in a 6-well plate and each well contained a piece of cellulose sponge (0.5×0.5×0.5 cm), 7 mL of M9 minimal medium (33.9 g/L Na₂HPO₄, 15 g/L KH₂PO₄, 5 g/L NH₄Cl, 2.5 g/L NaCl, and glucose at 0.4% (w/v)) or 50% LB (5 g/L tryptone, 2.5 g/L yeast extract, and 5 g/L NaCl) supplemented with 50 µg/mL of Amp and 25 µg/mL of Km. Diluted overnight cultures of BL21/(pSTB7+pYYDT) and BL21/(pSTB7+pYYDT-L) with a final OD_{600nm} of ~ 0.1 were inoculated into the 6-well

plates. The cultures were incubated at room temperature (25-28 °C) without shaking in the dark or irradiated with NIR light (660 nm, 1.33 mW/cm²).

4.2.4 CLSM and image analyses

Biofilms formed on the sponges were stained with 2.5 µM of SYTO 9 (a green fluorescent nucleic acid stain). To examine cell viability, biofilms were stained with 2.5 µM of SYTO 9 and 66.7 µM of propidium iodide (PI) using LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Molecular Probes, Inc.) (Teal et al., 2006). After staining for 20 min in the dark, the sponges were washed twice with 1× PBS (8.0 g/L NaCl, 3.23 g/L Na₂HPO₄·12H₂O, 0.45 g/L NaH₂PO₄·2H₂O, pH 7.0) to remove excess dyes. The biofilms were imaged using a confocal scanning microscopy (CLSM) (Carl Zeiss Microscopy LSM 780). All experiments were conducted with three biological replicates, and for each replicate, three images were taken at random locations. Biofilm biovolume and thickness were analyzed using IMARIS (version 8.2.0, Bitplane, Zurich, Switzerland).

4.2.5 Biotransformation of indole to tryptophan by planktonic cells

The biotransformation experiment with planktonic cells was conducted using a previously described method with slight modification (Perni et al., 2013). The overnight cultures grown in LB media at 37 °C were inoculated in M9 minimal media or 50% LB media. After 24-h incubation at room temperature (25-28 °C) in the dark or under the NIR light (660 nm, 1.33 mW/cm²), cells were harvested through centrifugation at 6,000 g. The pellets were washed three times with 1×PBS buffer. Then the PBS buffer was replaced by 20 mL of reaction buffer (0.1 M KH₂PO₄, 7 mM serine, 0.1 mM pyridoxal 5-phosphate

(PLP), 5% (v/v) DMSO, 2 mM indole, PH 7.0). The reaction mix ($OD_{600nm} \sim 2$) was incubated at room temperature with shaking (70 rpm) in the dark or under the NIR light.

4.2.6 Biotransformation of indole to tryptophan by biofilms

Milliliter-scale submerged biofilm reactors were set up in triplicates using the previously described method with slight modification (Ding et al., 2014). Ten-mL syringes (Luer-Lok tip, latex free; BD) were packed with cellulose sponge cubes ($1 \times 1 \times 1$ cm) (Scotch-Brite) for biofilm growth. Each reactor was inoculated using diluted overnight LB cultures ($OD_{600nm} \sim 0.1$) followed by a 1-h stop flow to allow cells to attach onto the sponge cubes. M9 medium or 50% LB containing 50 $\mu\text{g/mL}$ of Amp, 25 $\mu\text{g/mL}$ of Km and 7 mM of serine was then continuously fed at a flow rate of 8 mL/h for biofilm growth. Indole was introduced to the M9 medium and 50% LB medium at a final concentration of 2 mM after 84 h and 48 h, respectively. Tryptophan in the effluent from the submerged biofilm reactors were determined at predetermined time points. Cell detachment rate influenced by indole was quantified using a drop-plate method (Chen et al., 2003; Sivakumar et al., 2014; Wu et al., 2016). To study biofilm formation and biotransformation simultaneously and dynamically, M9 medium containing 50 $\mu\text{g/mL}$ of Amp, 25 $\mu\text{g/mL}$ of Km, 7 mM of serine and 2 mM of indole was continuously fed at a flow rate of 8 mL/h for biofilm growth and biotransformation. SYTO 9-stained biofilm and tryptophan in the effluent were then observed and determined at predetermined time points. M9 medium or 50% LB containing 50 $\mu\text{g/mL}$ of Amp, 25 $\mu\text{g/mL}$ of Km, 2 mM of indole and 7 mM of serine was then continuously fed at a flow rate of 8 mL/h for biofilm growth.

4.2.7 Quantification of indole, tryptophan and ammonium

Samples were filtered through 0.2 μm Acrodisc122 polyethersulfone membrane syringe filters (Pall Corporation, Singapore). The concentration of tryptophan was quantified using a HPLC (Shimadzu, Singapore) equipped with a ZORBAX (SB-C18 4.6 mm $\times$ 15 cm) column (Tsoligkas et al., 2011). A mixture of methanol (solvent A) and water (solvent B) was applied as the mobile phase (1 mL/min at 30 $^{\circ}\text{C}$) with controlled gradients as follows: 0-0.5 min, 0-10% A; 0.5-12.5 min, 10-90% A; 15-16 min, 90-10% A; 16-21 min, 10% A (Perni et al., 2013). The mobile phase was acidified with 0.1% formic acid. The elution time was around 8.2 min. Indole concentration was determined using the Ehrlich's reagent prepared by dissolving 2.0 g of *p*-dimethylaminobenzaldehyde (DMAB) in 50 mL of 95% ethanol with concentrated hydrochloric acid (Wang et al., 2017). Indole standards (0 to 1 mM) were prepared freshly in the reaction buffer for immediate use. Fifty μL of the standards or appropriately diluted samples was mixed with 150 μL Ehrlich's reagent and incubated for 15 min at room temperature followed by the measurement of an optical density at 530 nm. Ammonium concentration was determined using the Ammonia TNTplus Vial Test kit (Hach, USA).

4.3 Results and discussion

4.3.1 The design of NIR light responsive, tryptophan-producing biofilm

We introduced a previously constructed NIR light responsive c-di-GMP gene circuit (Hu et al., 2017) into *E. coli* so that the formation of the engineered *E. coli* biofilm could be modulated by the NIR light. The gene circuit is comprised of a bacteriophytochrome c-di-GMP synthase gene (*bphS*) (Ryu and Gomelsky, 2014) and a chromophore BV synthase gene (*bphO*) from *Rhodobacter sphaeroides* (Figure 4.1). Previous work has shown that

BphG1, a bacteriophytochrome from *R. sphaeroides*, contains the NIR light photoreceptor domain, however it has a low DGC activity for c-di-GMP synthesis (Tarutina et al., 2006). BphS is an engineered BphG1 and it has a higher DGC activity (Ryu and Gomelsky, 2014). Since BphS originated from *R. sphaeroides*, *bphO* from the same bacterium for BV synthesis was incorporated into the gene circuit. BphS has two main domains: PAS-GAF-PHY domain, a photoreceptor module binding to light-absorbing chromophores (BV); and GGDEF domain, a DGC output domain synthesizing c-di-GMP to enhance biofilm formation. The presence of NIR light induces a conformational change of the BV-bound PAS-GAF-PHY domain in BphS, which exposes its GGDEF domain to enhance biofilm formation by synthesizing c-di-GMP. In our previous work, we used a NIR light responsive c-di-GMP gene circuit to increase c-di-GMP and enhance biofilm formation of *S. oneidensis* (Hu et al., 2017).

Figure 4.1 The design of NIR light responsive, catalytic *E. coli* biofilm for biotransformation of indole into tryptophan. The pYYDT-L vector with the pBBR1 origin and Km antibiotic resistance was employed to express NIR light responsive c-di-GMP synthase, while the pSTB7 vector with the pBR322 origin and Amp antibiotic resistance was used to overexpress tryptophan synthase. The c-di-GMP gene circuit was used to modulate biofilm formation. The tryptophan synthase TrpBA from *S. enterica* catalyzes the biotransformation of indole and serine into tryptophan in the presence of a cofactor PLP.

With the growing interest in developing biofilm-based bioprocesses and applying the engineered single species biofilms for synthesis of fine chemicals, the investigation of engineering *E. coli* biofilm becomes attractive due to the wide industrial applications of *E. coli*. In this study, we aimed to engineer a NIR light responsive *E. coli* biofilm for the biotransformation of indole into tryptophan. To generate a NIR light responsive biofilm

catalyst, we used two plasmids in *E. coli*, i) the plasmid pYYDT-L with pBBR1 origin and Km antibiotic resistance that expresses a NIR light c-di-GMP gene circuit and ii) the plasmid pSBT7 that encodes a tryptophan synthase (TrpBA) from *S. enterica*. Recombinant tryptophan synthase TrpBA comprises two subunits associated as an $\alpha 2\beta 2$ tetramer and the reaction catalyzed by TrpBA is shown in Figure 4.2. When both plasmids are transferred simultaneously into *E. coli*, the engineered *E. coli* cells are capable of catalyzing the biotransformation of indole to tryptophan and sensing NIR light to enhance biofilm formation.

Figure 4.2 Tryptophan synthase mediated conversion of serine and (halo)indole (where X = F, Cl, Br) to (halo)tryptophan with PLP. PLP with serine yields Intermediate 1 by a dehydration process mediated the enzyme. Then, intermediate 2 is resulted from the

nucleophilic attack of indole on intermediate 1, and a subsequent hydrolysis releases the product halotryptophan and the cofactor PLP.

4.3.2 Activity of NIR light responsive c-di-GMP gene circuit

The activity of the NIR light responsive c-di-GMP gene circuit was assayed on Congo red agar plates based on the c-di-GMP-dependent curly fimbriae formation (Hammar et al., 1995; Römling, 2005). The strain BL21 /(pSTB7+pYYDT-L) expressing NIR light responsive BphS and tryptophan synthase TrpBA was incubated on the Congo red plates under NIR light (660 nm, 1.33 mW/cm²) or in the dark at room temperature for 36 h. Control experiments were carried out in parallel with the strain harboring pSTB7 and the empty pYYDT vector. The colony of BL21 /(pSTB7+pYYDT-L) with exposure to NIR light was more pigmented than that in the dark, suggesting more curly fimbriae present in the colony and a high intracellular c-di-GMP level. In contrast, the change of colony color in response to NIR light was not observed for the control strain harboring pSTB7 and empty pYYDT (Figure 4.3 A). The results of Congo red assay are indicative of the higher c-di-GMP level of BL21 /(pSTB7+pYYDT-L) under the NIR light. Since c-di-GMP levels are highly dynamic, in particular, during biofilm formation under the exposure of NIR light, a broadly adaptable c-di-GMP biosensor capable of monitoring c-di-GMP levels in real time would be required for future studies.

Figure 4.3 Activity assay of the NIR light responsive c-di-GMP gene circuit. (A) Colony morphology on Congo red agar plates. (B) Representative CLSM images of the biofilms and (C) estimated biofilm biovolume of BL21/(pSTB7+pYYDT) and BL21/(pSTB7+pYYDT-L) formed on the sponges after 36 h incubation. Experiments were conducted in triplicate, and for each replicate, three representative images were acquired and quantified. The scale bar represents 50 μ m. **** P < 0.0001, Student's t test.

To further examine whether biofilm formation was enhanced by NIR light, we conducted an assay in which the sponge cubes were placed in 6-well plates containing M9 medium. Under dark or when exposed to NIR light, no significant difference was observed for the biofilm of the control strain BL21/(pSTB7+pYYDT) in terms of biofilm biovolume ($1.31 \pm 0.32 \mu\text{m}^3 \mu\text{m}^{-2}$ vs $1.25 \pm 0.44 \mu\text{m}^3 \mu\text{m}^{-2}$). In contrast, the biofilm of BL21/(pSTB7+pYYDT-L) expressing the NIR light c-di-GMP synthase was greatly

enhanced by NIR light ($1.07 \pm 0.31 \mu\text{m}^3 \mu\text{m}^{-2}$ in the dark vs $2.72 \pm 0.54 \mu\text{m}^3 \mu\text{m}^{-2}$ in NIR light) (Figures 4.3B and C). Similar results were obtained when the assay was conducted using 50% LB medium (Figure 4.4).

Figure 4.4 Activity assay of NIR light responsive c-di-GMP module using 50% LB medium. (A) Confocal images of biofilm and (B) biofilm biovolume of BL21/(pSTB7+pYYDT) and BL21/(pSTB7+pYYDT-L) on sponges under dark or NIR light. The scale bar represents 50 μm . **** $P < 0.0001$, Student's t test.

Taken together, the results showed that, in the presence of NIR light, the NIR light responsive c-di-GMP gene circuit was effective in elevating the c-di-GMP level and enhancing biofilm formation of *E. coli* growing in minimal medium or rich medium, indicating the robustness of our gene circuit for biofilm modulation by light.

4.3.3 Biotransformation catalyzed by planktonic cells

The engineered strain BL21/(pSTB7+pYYDT-L) grew comparably well as the control strain in the presence or absence of NIR light in M9 medium and 50% LB (Figure 4.5).

Figure 4.5 Growth curves of BL21/(pSTB7+pYYDT-L) grown (A) in M9 medium or (B) in 50% LB medium in the presence and absence of NIR light.

The biotransformation catalyzed by planktonic cells were assayed using indole as substrates in the dark or under the NIR light. Depletion of indole by the BL21/(pSTB7+pYYDT-L) cells was observed within 3 h for both conditions (Figure 4.6). Depletion of indole by the BL21/(pSTB7+pYYDT-L) cells was observed within 3 h for both conditions (Figure 4.6). No substantial difference in the indole depletion rate was observed for the reactions in the dark and under the NIR light. At 1.5 h, $90 \pm 3\%$ of total indole were converted in the dark as compared to $85 \pm 2\%$ ($p > 0.05$) were converted by BL21/(pSTB7+pYYDT-L) irradiated by NIR light (Figure 4.7).

Figure 4.6 Biotransformation of indole into tryptophan using planktonic cells of the engineered strain BL21/(pSTB7+pYYDT-L). The cells constitutively express tryptophan synthase and the NIR light responsive c-di-GMP synthase. Concentration profiles of indole and tryptophan in the reaction solutions: (A) Cells pre-grown in M9 media and used as biocatalysts under dark condition; (B) Cells pre-grown in M9 media and used as biocatalysts under NIR light; (C) Cells pre-grown in 50% LB media and used as biocatalysts under dark condition; (D) Cells pre-grown in 50% LB media and used as biocatalysts under NIR light. Ammonium concentration profiles also shown in (C) and (D). Ammonium undetectable for conditions (A) and (B). Experiments were conducted in triplicate.

Figure 4.7 Percentage of indole depletion by BL21/(pSTB7+pYYDT-L) grown in M9 medium in the presence and absence of NIR light (1.33 mW/cm²).

The results suggest that NIR light does not influence the ability of the BL21/(pSTB7+pYYDT-L) cells to convert indole to tryptophan. Interestingly, a prolonged periods of reaction time resulted in a decreased concentration of tryptophan and a stoichiometric increase of indole concentration (Figure 4.6C and D), which could be attributed to the catalytic conversion of tryptophan back to indole by tryptophanase (TnaA) (Stewart and Yanofsky, 1986; Watanabe and Snell, 1972). The detection of increasing amount of ammonium ions, a by-product of the catalytic conversion of tryptophan to indole by TnaA, from 24 h to 36 h (Figure 4.6C and D) confirmed the occurrence of the reverse reaction.

4.3.4 Biofilm-mediated biotransformation in submerged biofilm reactors

To study the effect of the optogenetic control on the biofilm formation of BL21/(pSTB7+pYYDT-L) in submerged biofilm reactors, we observed the mature biofilms that were supplied continuously with M9 medium for 84 h under NIR light and in the dark. A cell viability of $73 \pm 7\%$ for the biofilms grown under the NIR light was obtained, while the biofilms grown in the dark had a cell viability of $49 \pm 6\%$ (Figure 4.8). It has been reported that elevated levels of c-di-GMP contributed to the increased stress tolerance of bacteria biofilm (Chua et al., 2015; Gupta et al., 2014). Therefore, the higher c-di-GMP levels of biofilms grown under NIR light could have enhanced the biofilms' resistance to the antibiotics in the M9 medium, which leads to a higher cell viability.

Figure 4.8 Effect of NIR light on biofilms in submerged biofilm reactors: (A) Representative CLSM images of biofilms, (B) estimated thickness and cell viability of biofilms in the presence and absence of NIR light. The scale bar represents 50 μm . (C) Tryptophan concentration in effluent from the reactor under NIR light and in the dark. **** $P < 0.0001$, Student's t test.

The biofilm exposed to NIR light was substantially thicker than that incubated in the dark ($36.39 \pm 2.80 \mu\text{m}$ vs $23.51 \pm 2.34 \mu\text{m}$) (Figure 4.8B). As NIR light activated the DGC activity of BphS, NIR light increased c-di-GMP level to enhance biofilm formation.

When the biofilms were exposed to indole, a higher cell detachment rate from biofilms was observed under both NIR light and dark conditions (Figure. 4.9). It has been reported that temperature affects the role of indole in *E. coli* biofilm formation by regulating gene expression (Lee et al., 2008). One study has shown that indole concentration ranging from 0 to 1.25 mM increased biofilm formation of *E. coli* at 37 °C (Di Martino et al., 2003). However, other studies have shown that at 30 °C, an indole concentration of 0 to 2 mM decreased biofilm formation of *E. coli* instead (Domka et al., 2006; Lee et al., 2007), which is consistent with our results indicating that indole acts as a signal leading to decreased biofilm formation. This could be due to similar experimental conditions, including temperature and indole concentration (25-28 °C and 2 mM in our experiments, respectively).

Figure 4.9 Cell detachment rate from biofilms in the absence and presence of NIR light before and after 6-h exposure to indole. * $P < 0.05$, Student's *t* test.

Intriguingly, NIR light substantially reduced cell detachment from the biofilms (5.74×10^5 cells/h under NIR light vs 9.35×10^6 cells/h in the dark before indole exposure; 1.01×10^6 cells/h under NIR light vs 1.90×10^7 cells/h in the dark after indole exposure). It is because the NIR light-increased c-di-GMP level enhances biofilm formation (Figure 4.8B), resulting in a higher retention of cells in the biofilm reactors and the reduced cell detachment from the biofilms in the presence of NIR light. Taken together, our results show that NIR light enhances biofilm formation of the engineered light-responsive bacteria strain, increases cell viability and reduces cell detachment from the biofilms.

We further quantified tryptophan production by the biofilms of BL21/(pSTB7+pYYDT-L) grown in the dark and under NIR light. Interestingly, although the biofilms under the NIR light have more biomass than those in the dark, the concentration of tryptophan in the effluent from the biofilm reactors under the NIR light was substantially lower than that in the dark (Figure 4.8C). This could be attributed to high mass transfer limitation of substrate or product in an overly thick biofilm, resulting in a high amount of unproductive biomass at the bottom of the biofilm and hence a low specific catalytic activity (Stewart, 1998, 2003). A catalytic biofilm should ideally contain an appropriate amount of biomass with sufficient catalytic activity and a low mass transfer limitation.

To determine the optimal biofilm thickness for efficient biocatalysis, we quantified the biotransformation performance of growing biofilms. The dynamics of biofilms growing in M9 medium containing indole under both dark and NIR light conditions were quantified using CLSM imaging analyses (Figure 4.10A). The thickness of 36-h biofilm grown under the NIR light was significantly higher than that in the dark ($17.41 \pm 2.72 \mu\text{m}$ vs $12.01 \pm$

2.28 μm ; $p < 0.01$) (Figure 4.10B). As the NIR light continuously activated the DGC activity of BhpS, the thickness of the biofilm under NIR light increased from 22.36 ± 3.71 μm at 60 h to 32.01 ± 3.96 μm at 84 h. In contrast, the biofilm thickness under dark condition was 16.38 ± 3.32 μm at 60 h and 21.65 ± 7.07 μm at 84 h. Although indole reduces biofilm formation of *E. coli* under our experimental conditions (Figure 4.9), NIR light can still increase biofilm thickness in the presence of indole, suggesting that the engineered catalytic biofilm can overcome the inhibition of indole on biofilm formation by *E. coli*.

Figure 4.10 Biotransformation of indole into tryptophan by NIR light responsive biofilm in submerged biofilm reactors continuously supplied with M9 medium: (A) Representative CLSM images of the biofilms, (B) estimated biofilm thickness, (C) tryptophan concentration in effluent, (D) tryptophan concentration normalized to the biofilm

thickness, and (E) the amount of tryptophan produced by the biofilms in the presence and absence of NIR light. The scale bar represents 50 μm . ** $P < 0.01$ and *** $P < 0.001$, Student's t test.

From 36 h to 72 h, tryptophan concentration in the effluent from the reactor under NIR light was substantially higher than that from the reactor in the dark (Figure 4.10C), suggesting a higher conversion efficiency of the light responsive biofilm with the exposure to NIR light. However, from 72 h to 84 h, the concentration of tryptophan produced by the BL21/(pSTB7+pYYDT-L) biofilm under NIR light exhibited a $\sim 34\%$ decrease while we observed a $\sim 20\%$ increase in the concentration of tryptophan produced by the biofilm in the dark, which was not positively correlated with the biofilm thickness shown in Figure 4.10B. It could be attributed to an overly thick BL21/(pSTB7+pYYDT-L) biofilms at 72 h and 84 h in the presence and absence of NIR light, respectively. To determine the optimal biofilm thickness, we normalized the concentration of tryptophan produced by the BL21/(pSTB7+pYYDT-L) biofilms in the presence and absence of NIR light to the corresponding biofilm thickness in Figure 4.10B. Figure 4.10D shows that the biofilm thickness-normalized concentrations of tryptophan produced by biofilms under both conditions at 60 h were higher than those at other time points, suggesting that the biofilms with optimal thickness ranging from $16.38 \pm 3.32 \mu\text{m}$ to $22.36 \pm 3.71 \mu\text{m}$ formed at 60 h under our experimental conditions. Over a continuous operation of biofilm reactor for 96 h, $0.43 \pm 0.02 \text{ mmol}$ of tryptophan was produced in the presence of NIR, which was $\sim 30\%$ higher than that in the absence of NIR light ($0.36 \pm 0.01 \text{ mmol}$).

The amount of tryptophan produced by the biofilm pre-grown for 48 h under NIR light was slightly higher (~8%) than that in the absence of NIR light when using 50% LB medium (Figure 4.11A). In contrast, NIR light led to a 25% increase in tryptophan amount when indole concentration was increased from 2 to 4 mM (Figure 4.11B). This could be because an increase in the substrate concentration might result in an increased diffusion depth and reduce the amount of unproductive biomass. Our results highlight the need to maintain an optimal range of biofilm thickness for robust biofilm-mediated chemical synthesis and the need for a bi-directionally controllable thickness of catalytic biofilms (Mukherjee et al., 2018).

Figure 4.11 (A) and (B) The amount of tryptophan produced by the biofilms in the presence and absence of NIR light when the concentrations of indole are 2 mM and 4 mM, respectively.

4.4 Conclusion

Biofilm as a living catalysts has been exploited for the production of biohydrogen, biofuels, and electricity in microbial fuel cells as well as in the synthesis of bulk and fine chemicals (Botyanszki et al., 2015; Cologgi et al., 2014; Li et al., 2013; Liu et al., 2015).

However, the structure and activity of biofilms are highly dynamic and heterogeneous, which makes the control of biofilm-mediated bioprocesses challenging and limits their application in industrial biotechnology. To address these challenges, genetic reprogramming of bacterial biofilms is a promising strategy. Here we show that a NIR light responsive biofilm was achieved by constructing a NIR light responsive gene circuit to artificially override the endogenous c-di-GMP signaling network. This is the first report on using a light responsive biofilm for chemical synthesis and it demonstrates the feasibility of applying light to “guide” biofilm formation and improve catalytic performance for chemical production. Since c-di-GMP regulates biofilm formation and dispersal in a wide variety of bacteria, the optogenetic approach reported here is expected to be compatible with more complex metabolic engineering in other bacteria for chemical synthesis.

Chapter 5. **Engineering a light-responsive, quorum quenching biofilm to mitigate biofouling on water purification membranes**

*This chapter has been published as Manisha Mukherjee, Yidan Hu (co-first author), Chuan Hao Tan, Scott A. Rice, Bin Cao (2018) Engineering a light-responsive, quorum quenching biofilm to mitigate biofouling on water purification membranes. **Science Advances** 12: eaau1459. Permission has been granted by the licensed content publisher “Science” to use the published content as a chapter in this thesis.*

5.1 Introduction

As the global demand for freshwater increases, membrane technology has emerged as one of the leading approaches for water treatment from lower-quality water sources (Shannon et al., 2008). In recent years, FO technology has been applied to various water treatment processes because of its lower energy consumption and lower fouling propensity than pressure-driven approaches such as nanofiltration and reverse osmosis (Achilli et al., 2009; Lee et al., 2010; Zhao et al., 2012). Although FO processes tend to foul less than pressurized membrane processes, biofouling, i.e., undesirable attachment and growth of microorganisms on membranes, still remains as one of the most important challenges in practical applications of FO (Flemming et al., 1997; Kim et al., 2014).

In biofouling, cells form a structured microbial consortium on membrane surfaces known as biofilms, in which cells are embedded within a self-produced extracellular matrix

(Flemming et al., 1997; Komlenic, 2010). QS, i.e., a chemical-based intercellular communication between bacterial cells, has been shown to play an important role in biofilm formation (Davies et al., 1998; Labbate et al., 2004). Hence, inhibiting QS system or QQ has been reported to be a promising approach for biofouling control (Davies et al., 1998; Labbate et al., 2004). Recent studies have shown that AHL-based QS was involved in membrane biofouling, and inactivation of QS by enzymes that degrade AHL substantially reduced biofouling (Kim et al., 2013; Kim et al., 2013; Yeon et al., 2009). Purified QQ enzymes are often of high cost and low stability; in contrast, QQ bacteria that continuously produce AHL-degrading enzymes have attracted great interest because of the cost benefits and their continual production of QQ enzymes (Oh et al., 2017; Oh et al., 2012; Weerasekara et al., 2014; Yu et al., 2016). However, entrapment of QQ bacteria in porous matrices such as hydrogels or hollow fibers (Kim et al., 2013; Oh et al., 2012) is required to retain them in continuously operated membrane processes and to prevent uncontrollable biofilm formation by the QQ bacteria on membrane surfaces. It would be more desirable if the formation of the biofilms by QQ bacteria on membranes could be controlled so that the QQ biofilm itself does not compromise membrane performance. Unfortunately, controlling biofilm development through adjusting physicochemical parameters alone is highly challenging because of the interplay between the dynamic, sophisticated, biofilm signaling networks and heterogeneous microenvironments within biofilms.

Biofilm formation and dispersal in a wide variety of bacteria is controlled by a signal cascade mediated by the intracellular secondary messenger c-di-GMP. Typically, high c-di-GMP levels promote biofilm formation, while low levels lead to reduced biofilm formation and increased dispersal (Romling et al., 2005; Valentini and Filloux, 2016).

Hence, modulating the intracellular c-di-GMP level is a promising strategy toward a controllable biofilm development (Benedetti et al., 2016). In this study, we engineered a biofilm of *Escherichia coli* by introducing synthetic gene circuits to control its own biofilm formation by modulating intracellular c-di-GMP concentration and to inhibit biofouling caused by other bacteria via production of QQ enzymes. To control biofilm formation, we introduced NIR (660 nm) light-responsive DGC (c-di-GMP synthase) activity (Ryu and Gomelsky, 2014) and blue (465 nm) light-activated PDE (c-di-GMP hydrolase) activity into *E. coli*. To enable the production of a QQ enzyme that degrades AHL-based QS signals, we introduced the gene *aiiO* (Oh et al., 2017) into the *E. coli* strain. Following this, an *E. coli* biofilm with a light-responsive thickness and biovolume was achieved on FO membranes, and this biofilm effectively inhibited biofouling caused by a model organism.

5.2 Materials and methods

5.2.1 Bacterial strains and culture conditions

All strains and plasmids used in this study are summarized in Table 5.1. All strains were routinely maintained in LB at 30 °C. Antibiotics were supplemented into the growth media whenever necessary [Amp (100 µg/mL), Km (50 µg/mL) and Gm (80 µg/mL); Sigma-Aldrich]. Stock cultures were maintained in LB medium with 20% glycerol at -80 °C. Seed culture was prepared by transferring 0.5 mL of stock culture to 50 mL LB medium in a 200-mL Erlenmyer flask and incubated for 12 h at 30 °C with shaking at 200 rpm.

Table 5.1 Bacterial strains and plasmids used in this study

Bacterial strains	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
DH5 α	The strain for gene cloning	Thermo Fisher Scientific
BL21(DE)	The strain for protein expression	Thermo Fisher Scientific
<i>Pantoea stewartii</i>		
<i>P. stewartii</i> R067d	AHL producer and model biofouling organism	(Tan et al., 2015)
<i>P. stewartii</i> YFP	Gm ^R , <i>yfp</i> , AHL producer	This study
Plasmids		
pYYDT	Km ^R ; <i>oriV</i> (pBBR1), <i>Ptac</i>	(Yang et al., 2015)
pYYDT-L	Km ^R ; <i>oriV</i> (pBBR1), <i>Ptac</i> , <i>bphS</i> , <i>bphO</i>	(Hu et al., 2017)
pMal-EB1	Amp ^R ; <i>oriV</i> (pBR322), <i>Ptac</i> , <i>eb1</i>	(Ryu et al., 2017)
pYYDT-RB	Km ^R ; <i>oriV</i> (pBBR1), <i>Ptac</i> , <i>bphS</i> , <i>bphO</i> , <i>eb1</i>	This study
pTrcHis2-aiiO	Amp ^R and Km ^R ; <i>oriV</i> (pBR322), <i>Ptrc</i> , <i>aiiO</i>	(Oh et al., 2017)
pTrcHis2-maiiO	Amp ^R and Km ^R ; <i>oriV</i> (pBR322), <i>Ptrc</i> , <i>maiO</i>	This study
pTrcHis2-aiiO.mCherry	Amp ^R and Km ^R ; <i>oriV</i> (pBR322), <i>Ptrc</i> , <i>maiO</i> ; <i>PguaB</i> , <i>mCherry</i>	This study
pTrcHis2-maiiO.mCherry	Amp ^R and Km ^R ; <i>oriV</i> (pBR322), <i>Ptrc</i> , <i>maiO</i> ; <i>PguaB</i> , <i>mCherry</i>	This study
pAiiO	Amp ^R ; <i>oriV</i> (pBR322), <i>Ptrc</i> , <i>aiiO</i> ; <i>PguaB</i> , <i>mCherry</i>	This study
pmAiiO	Amp ^R ; <i>oriV</i> (pBR322), <i>Ptrc</i> , <i>maiO</i> ; <i>PguaB</i> , <i>mCherry</i>	This study

5.2.2 Plasmid construction and transformation

All plasmid constructions were conducted in *E. coli* DH5 α through transformation using the heat shock method. The chemically competent *E. coli* cells were prepared using the Inoue method (Sambrook and Russell, 2006). To dynamically control biofilm formation and dispersal, a bidirectionally controllable c-di-GMP module, comprising a previously constructed NIR light-responsive c-di-GMP gene module (i.e., *bphS* and *bphO*) (Hu et al., 2017; Ryu and Gomelsky, 2014) and a blue light-activated PDE gene *eb1* (Ryu et al.,

2017), was constructed as follows. The gene *ebI* with a *Ptac* promoter and the Shine-Dalgarno (SD) sequence was amplified by PCR from pMal-EB1 using primers Blue-Ptac-F (5'-GGACTAGTTGACAATCATCGGCTCGTAT-3'), and Blue-Ptac-R (5'-GGACTAGTTCATGAGTCCAGACTGATGGTTC-3') (Spe I enzyme site underlined). Spe I-digested fragment was ligated with the pYYDT-L fragment, which was cut using Spe I for fragment ligation and dephosphorylated using FastAP thermosensitive alkaline phosphatase, forming the plasmid pYYDT-RB. The constructs were selected by colony PCR using YYD-F (5'-GCCTCAGGCATTTGAGAAGCACA-3') and YYD-R (5'-AGAGCGTTTACCGACAAACAACAGATAA-3') and further verified to confirm the orientation of *ebI* by DNA sequencing (AIT biotech, Singapore). In pYYDT-RB, *bphS* and *bphO* were placed under *Ptac* as an operon, whereas *ebI* with an additional *Ptac* promoter was inserted downstream of the *bphS-bphO* operon to achieve coordinated efficient expression of all genes. The gene *aiiO* encoding a QQ enzyme (Oh et al., 2017) was then combined with bidirectionally controlled c-di-GMP module in *E. coli* BL21(DE). A 99-base pair (bp) fragment, flanked by Nru I restriction sites at each end, was amplified by PCR from the multiple cloning sites of pUCP22 (Herrero et al., 1990; West et al., 1994). The Nru I-digested 99-bp fragment was inserted at an Nru I site, near the C-terminal, of *aiiO* to generate an *aiiO* mutant (i.e., *maiO*) that does not produce QQ enzyme. The *aiiO* gene (Oh et al., 2017) and its mutant *maiO* were cloned into pTrcHis2-*aiiO* and pTrcHis2-*maiO*, respectively, with Amp- and Km-resistant markers (Oh et al., 2017). The *mCherry* gene with a *PguaB* promoter and the SD sequence, flanked by Pme I restriction sites at each end, was amplified by PCR from pABG5:*PguaB*-*mCherry* (from Garry Port/Caparon lab). The Pme I-digested *PguaB.mCherry* fragment was inserted at a Pme I site downstream

of the kanamycin resistance gene in both pTrcHis2-aiiO and pTrcHis2-maiiO to construct the pTrcHis2-aiiO.mCherry and pTrcHis2-maiiO.mCherry, respectively. To enable the compatibility of pTrcHis2-aiiO.mCherry and pTrcHis2-maiiO.mCherry with pYYDT-RB in a stable double plasmid system, the kanamycin resistance genes flanked by two Hind III restriction sites at each end were removed using Hind III restriction enzyme, and then pTrcHis2-aiiO.mCherry and pTrcHis2-maiiO.mCherry were recircularized by T4 ligase to generate the resulting plasmids pAiiO and pmAiiO. pYYDT-RB and pAiiO (or pmAiiO) were simultaneously transformed into *E. coli* BL21(DE). The sequence details for the constructed gene circuits are included in table 5.2.

Table 5.2 Sequence of gene circuits

<i>Ptac</i>-RBS-<i>eb1</i>(2483bp)
<p><i>Ptac</i>(TGACAATTAATCATCGGCTCGTATAATGT)GTGGAATTGTGAGCGGAT AACAATTTACACAGGAAACAGCCAGTCCGTTTAGGTGTTTTCACGAGCAC TTCACCRBS(AACAAGGACC)ATAGCAT<i>eb1</i>(atgaaaatcgaagaaggtaaactggaatctggat taacggcgataaaggctataacggtctcgtgaagtcggtaagaaatcgagaaagataccggaattaaagtcaccgttgag catccggataaactggaagagaaattcccacaggttcgggcaactggcgatggccctgacattatcttctgggcacacgacc gctttgggtggctacgctcaatctggcctgttggctgaaatcaccccgacaaagcgttcaggacaagctgtatccgtttacct gggatgccgtacgttacaacggcaagctgattgcttaccgatcgtgtgaagcgttatcgtgatttataacaaagatctgct gccgaacccgccaaaaacctgggaagagatcccgcgctggataaagaactgaaagcgaaaggtgaagagcgcgctgat gttcaacctgcaagaaccgtacttcacctggccgctgattgctgctgacgggggtatgcgttcaagatgaaaacggcaagt acgacattaaagacgtggcggtggataacgctggcggaagcgggtctgaccttctggttgacctgattaaaaacaaaca catgaatgcagacaccgattactccatcgagaagctgcctttaataaggcgaaacagcgatgacctcaacggccccgtg ggcatggtccaacatcgacaccagcaaaagtgaattatggtgtaacgggtactgccgaccttaagggtcaacctccaaccg ttcgttggcgtgctgagcgcaggtattaacgccgccagtcggaacaaagagctggcaaaagagttcctcgaaaactatctgc tgactgatgaaggtctggaagcggtaataaagacaaaccgctgggtgccgtagcgtgaagtcttacgaggaagagttgg cgaaagatccacgtattgccgccactatggaaaacgcccgaaaggtgaaatcatgccgaacatcccgcatgtccgcttt ctggtatgccgtgctgactcggtgatcaacgccgccagcggctcgcagactgctgatgaagccctgaaagacgcgcaga ctaattcgagctcgaacaacaacaataacaataacaacacctgggtagcagggaaggatttcagaattccatcatcat catcatcacgaaaacctgtatttcagagcatgccagattacaaggatgacgatgacaaggcaaggcaggtgcttctcttgc gacatggccatcattcagtaaatgaagaggggattattattgaagtaaaccgcttgcctctgaaatgttcggttattctgaaca agaaatgatcgcccttctgtccacttctttacctccgattccgaaaacgccacaccttactatatcaaggatttgatagtg tgaagtgcagagcgcagaatggggcaaaagaggtgaggtcacagggtatcgtaaagatggtaccttctccctgttgaagc ctctattgcaaaagtatatgatgcaatgaaaaatgggtgatggttgcgtcatttagggagttgactgaactaaacgagcacaaga agagcttgtcgtcgtgccacccatgatccttaacgggcctgccaatcgtactctattcagagagcgttaacgagtgctct</p>

ccataggtcacatcaaaatggtgagaacattggtctcttttatcaacctggatggcttcagagagattaacgactcttatgggc atgagatgggagatctccttttagaagaggtttctcaaaggtgatcacagtgggtgaaccagggagacacggttggtcggtta gcaggggatgagtttatcattttatgtgaacacatacagaatccggagcctctctcatctttggctcgagagattacgactcttat gggcatgagatgggagatctccttttagaagaggtttctcaaaggtgatcacagtgggtgaaccagggagacacggttggtc ggtttagcaggggatgagtttatcattttatgtgaacacatacagaatccggagcctctctcatctttggctgtatcagtcaccaat gtgatccgtgagcctattgtccatgatgattgaaactgcgtgtgaccgcaagtataggcatcgcccttgggcatggctcaaca catagtgcagatgatttgatgcgctctgctgactccgccatgaacagtgttaaggagcgaggctgcaatgggtgggagttttc aatccaacgctgcaagaagaggctgaaaagcggctcgcaatctcaacaggctctcgcacagccattgataacaacgagttt acgctcgttaccacacctattgtggattctgagtcgggaatgattcgtggtgctgaactcttgggtgcgatggcggtcatcggtgg tgaaatctcgccaggcgtctttatccccgttccgagatgacaggtgctatcatcaccatcgggaggtgggttttgaagaagg gtgtaaagcagagagaagatggaaagccaaatttggggagcaagccccatatatcacgataaacgtctccacacgccagct atcagagccacttattattgaagagttccaggaaattctggctgaaaccgggtgcagatccaacacgtatcattttagagatcact gaaacatcattgatggcagatgttcagcaaacagggaagtgttaaacagggttggttacaactggaatgagagtagctgtgg acgattttggtacgggctactcatcccttgcacagctttcacgccttaaggtaaacgctgaaaattgatcgtgagtttgctg ggattagatgagggagaagaggacagattgtgtctctgccattagcaggctggctaaatcacttaaaccaagattgttgca gaaggtgttgaaacggatgagcaaaaggaacactatccgttaatcggtatgcgatctgatccaaggctactattaccaccgc cattatcagaagaagcactttttgaagctgttagcactagcttgagtcagaagaaccacaatgaacagcctttgaagttcttgatt tatattagtcgccctaagtttgagataactgaggaaggcctgcaggcaattttgaagcaatcaagagaattcaacctgaaaagt ggaatcacaggctatctttctatctgaaaggtgcgtttgcgcaataacctggaaggggatgaagagcctttgagccaactttatc aatcaattcaaaaaagacaataggcacagtatttaagatcattgcagagggagatctccaaccagactctttgctggctgg gaaatgggatacaaaactagacgggtgattgctttctgatagagccaatcttagtcagaacgaatcgataattatgagtgg ctgagcaggcaccagagctgtgctgtgaaatctttgaaaccatcagctctggactcataggaagct)
<i>Ptrc-aiiO</i> (919bp)
<i>Ptrc</i> (caatctgtgtgggcactcgaccggaattatcgattaactttattattaaaaattaaagaggtatatattaatgtatcgattaa at)aaggaggaataaaccatggccctt <i>aiiO</i> (atgaaatcccatgaaatcgagaccagccacggctcgattgccatccgtga aagcaggggtaacggaacgcccttgctgatgatccatggcaactccagcgcaggcgccatctttgcgccccagcttgaggg cgaaatcggtagaaactggcgggtgatcgcgccagatttgccggggcatggccagtcggcgacgcccttgatccggatc gcagctattccatggaaggctatgccgacgcgatgacggaagtgcctggcgaagctcggcatttcggatacagtcgtttcgg ctggtcgcttggcggccatatcggcacgcagatgatttcacgctttccggcatgcgcggcctgatgattaccggcacgccg cctgtagcgcgtgaggaagtggggcagggatttaagagcgggtccagatatggcacttgccgggcaggaagtctttccgac cgcgatgtcgaatcctatgcacgcagtacctgcggagaaccggttcgaagaccagttgcttgagattgtcgccgcacggatg ggcgcgcgcgccgatcatgttcgaaaaatttcagccggaaccggcggaaccagcgcgatattgttgcgaagcaagg ctcccattgcagttgtcaacggctcgcgaagagccgtttgtcgaacttgatttcgtctcgaaagtcggttcggcaatctctggg aaggtaaaaccatgtcatcgacggggcagggcatgcgcccttcgcgagacaccagccgtcttcgacgattatctgcagc gcttcatgcgcgactgcacggcctga)
<i>maiO</i> (909bp)

```

atgaaatcccatgaaatcgagaccagccacggctgcattgccatccgtgaaagcaggggtaacggaacgcccttgctgatg
atccatggcaactccagcgcaggcgccatctttgcgcccagcttgaggcgaaatcggtagaaactggcgggtgatcgc
gccagatttgccggggcatggccagtccggcgacgcccttgatccgcatcgcagctattccatggaaggctatgccgacgc
gatgacggaagtctggcgaaagctcggcatttcggatacagtcgtttcggctggctgcttggcgccatcggcatcgag
atgatttcacgctttcccgcatgcgcggcctgatgattaccggcacgccgctgtagcgcgtgaggaagtggggcagggga
tttaagagcgggtccagatatggcacttgccgggcaggaagtctttccgaccgcgatgtcgaatcctatgcacgcagtacctg
cggagaaccgttcgaagaccagtgtgcttgagattgtgcgccgcacggatggggcgcgcgggcgcatcatgttcgaaaaattt
gcagccggaaccggcggaaccagcgcgatattgttgcgaagcaaggctcccattgcagttgtcaacggtcgcgacca
gtcacgacgttgtaaaacgacggccagtgccaaagctggggcgccgcaagcttgcatgcctgcaggtcgactctagaggat
ccccgggtactcggaagagccgtttgtcgaacttgatttcgtctcgaaagtcggttcggcaatctctgggaaggtaaaacc
atgtcatcgacggggcagggcatgcgcccttcgcgagacaccagccgtcttcgacgattatctgcagcgcttcacgcgcg
actgcacggcctga

```

5.2.3 Congo red assay

To examine the synthesis of curly fimbriae as a proxy for the c-di-GMP level, strains were grown on LB agar media supplemented with Congo red (50 µg/mL) at 25 °C for 24 h and incubated in the dark or irradiated with NIR light (660 nm, 5.3 mW cm⁻²) or blue light (465 nm, 5.3 mW cm⁻²; pulses of 15 s light, 60 s dark) (Fang and Gomelsky, 2010).

5.2.4 Biofilm formation assay

Biofilm assay was conducted on the basis of a 24-well plate method with slight modifications (Ding et al., 2014; Wakimoto et al., 2004). Diluted overnight cultures of *E. coli*/pYYDT and *E. coli*/pYYDT-RB, at final turbidity of OD_{600nm} (optical density at 600 nm) ~ 0.1, were inoculated into 24-well plates with modified M9 minimal media. The cultures were incubated for 24 h at 25°C without shaking in the dark or irradiated with NIR light or blue light. After the crystal violet was added to each well, the wells were rinsed and dried, and then ethanol was added to dissolve the crystal violet. The total biofilm formation was measured at 550 nm. At least nine independent replicates were used for each strain.

5.2.5 AHL degradation assay

The QQ activity of *E. coli*/(pYYDT-RB + pAiiO) and *E. coli*/(pYYDT-RB + pmAiiO) was tested as follows. Two milliliters of bacterial culture in modified M9 minimal medium was collected at 0, 2, 6 and 12 h. The cell pellet was collected after being centrifuged at 8000 g for 10 min, while the cell supernatant was filtered through a 0.2 μ m filter (Polyethersulfone, Sartorius) and treated as the cell-free supernatant sample. 3-Oxo-hexanoyl-homoserine lactone (3OC6-HSL) was used as a model AHL signal and added into the cell pellet, which has been resuspended in fresh modified M9 medium, and cell-free supernatant, to achieve a final concentration of 1 μ M. The mixtures were incubated at 25 °C with constant shaking at 100 rpm for 2 h. After centrifugation and ultraviolet sterilization, residual 3OC6-HSL in each sample was measured using the reporter strain *E. coli* JB525 (Andersen et al., 2001). The green fluorescence intensity was quantified using a microplate reader (Tecan Infinite M200) at excitation and emission wavelengths of 488 nm and 530 nm, respectively.

5.2.6 Extraction and quantification of c-di-GMP

The extraction and quantification of c-di-GMP followed established methods (Wu et al., 2015). The light-responsive strain, *E. coli*/pYYDT-RB was grown at 25 °C in modified M9 minimal medium [33.7 mM Na₂HPO₄, 22.0 mM KH₂PO₄, 8.55 mM NaCl, and 9.35 mM NH₄Cl supplemented with 1/10-strength LB (pH 7.0)] in the dark, NIR light (660 nm, 5.3 mW cm⁻²) and blue light (465 nm, 5.3 mW cm⁻²; pulses of 15 s light and 60 s dark). At late exponential growth phase, 15 mL of culture was collected by centrifugation at 10,000 g for 5 min at 4 °C for c-di-GMP extraction using organic solvents (Kuchma et al., 2010).

The concentration of c-di-GMP was determined by using a Thermo Accela 1250 series liquid chromatography system with a Thermo Velos Pro Orbitrap mass spectrometer, as described earlier (Spangler et al., 2010). The c-di-GMP concentration was normalized to total protein quantified by Qubit protein assay.

5.2.7 Dynamics of the light-responsive biofilm on FO membrane

Biofilm development on water-purifying FO membranes was performed in an in-house-developed, CLSM-compatible membrane flow cell as described previously with some modifications (Mukherjee et al., 2016). The commercial FO membrane, Aquaporin-TFC was obtained from Sterlitech Corporation. The thickness of the membrane varied from 95 to 125 μm . Three individual membrane coupons with an effective area of 460 mm^2 for each were embedded in the different channels of the flow cell. The fully assembled membrane flow cell was sterilized with recirculation of 70% ethanol for 1 h. The flow cell was further flushed with autoclaved, deionized water to remove the ethanol from the system before commencing the biofouling experiment. The feed solution was composed of modified M9 minimal media, while the draw solution was 1 M NaCl (Mukherjee et al., 2016). The system was stabilized and the membranes were conditioned for 24 h to obtain the initial water flux before injecting the bacterial culture into the feed solution. To maintain the osmotic pressure gradient, fresh feed and draw solutions were added into the reservoirs daily at a rate of 50 mL/day to ensure that the exchange of liquid between the two chambers did not interfere with the measurement of permeate water flux and that the system could be operated at a constant osmotic pressure driving force during the experiment. The flow rate of the recirculation on both sides of each membrane was set to

2 mL/h. Overnight cultures of the light-responsive strain (*E. coli*/pYYDT-RB) and the control strain (*E. coli*/pYYDT) were added to the respective feed solution to a final OD_{600nm} of ~ 0.1. Biofilms were initially grown for 48 h in the dark, and then the 48-h biofilms on FO membranes were subjected to sequential irradiation by NIR light and blue light. The biofilm was observed every 24 h using CLSM, and the transmembrane water flux was recorded during the entire biofouling experiment.

5.2.8 Biofouling dynamics of *P. stewartii* in the presence of the engineered QQ biofilm

The biofilms of *E. coli*/(pYYDT-RB + pAiiO) and *E. coli*/(pYYDT-RB + pmAiiO) were grown on the FO membranes for 24 h in the dark. After 24 h, fresh feed solution was inoculated with an overnight *P. stewartii* culture to a final OD_{600nm} of ~ 0.1 and monitored for 96 h. Biofilm formation by *P. stewartii* was compared for membranes without the preformed *E. coli* biofilms. To regulate the QQ biofilm formation, the biofilms on FO membranes were subjected to sequential irradiation by NIR light and blue light, as described above. Biofilms were visualized every 24 h using CLSM, and the permeate flux was recorded during the entire biofouling experiment.

5.2.8 Confocal microscopy and imaging

Biofilms grown in the membrane flow cell were monitored using a CLSM (Carl Zeiss Microscopy LSM 780). The yellow fluorescent protein (YFP)-tagged *P. stewartii* was observed with excitation and emission at 514 and 527 nm, respectively. The mCHERRY-tagged *E. coli* was observed with excitation and emission at 550 and 650 nm, respectively. The CLSM images were analyzed using Imaris (Bitplane, Zurich,

Switzerland) to obtain quantitative parameters such as biovolume and thickness (Mohanty et al., 2015; Wu et al., 2015b). To estimate the biofilm biovolume and thickness, five regions on each membrane were randomly picked from the center of the channel for image acquisition, which is a common practice in flow cell-based biofilm studies (Heydorn et al., 2000; Korber et al., 1993). At least three independent replicates were used for each experiment.

5.3 Results and discussion

5.3.1 Design of the engineering gene circuits

To form an antifouling biofilm layer on FO membranes, we aimed to develop an engineered *E. coli* biofilm that itself does not attain a large thickness but is capable of inhibiting biofilm formation of other bacteria. C-di-GMP is an important secondary messenger involved in biofilm regulatory networks that coordinates biofilm formation and detachment in a wide range of bacteria (Hengge, 2009). Earlier studies have shown that increased c-di-GMP promotes biofilm formation, whereas a decrease leads to reduced biofilm formation and increased dispersal (Boyd and O'Toole, 2012; Romling et al., 2005). In general, c-di-GMP levels are controlled by two groups of enzymes: i) DGCs that synthesize c-di-GMP and ii) PDEs that hydrolyze c-di-GMP into two GMPs (Borlee et al., 2010; Valentini and Filloux, 2016). In this study, we engineered a bacterial biofilm whose growth and dispersal can be modulated by light through implementing a dichromatic, optogenetic c-di-GMP gene circuit (Ryu et al., 2017), in which the bacterial cell senses NIR light and blue light to adjust its c-di-GMP level. To do so, we first introduced a synthetic NIR light-responsive c-di-GMP module into *E. coli* BL21 (Hu et al., 2017; Ryu and Gomelsky, 2014). The module consisted of an engineered protein, BphS, a

bacteriophytochrome c-di-GMP synthase, and BphO, a chromophore BV synthase from *Rhodobacter sphaeroides* (Ryu and Gomelsky, 2014). In the presence of NIR light, the BV-bound photoreceptor domain (PAS-GAF-PHY) in BphS undergoes conformational changes and activates the DGC output domain (GGDEF), thereby synthesizing intracellular c-di-GMP to enhance biofilm formation (Figure 5.1A). In our previous study, the NIR light-responsive activity in *Shewanella oneidensis* showed an increase in c-di-GMP and enhanced biofilm formation (Hu et al., 2017). However, a constantly high DGC activity could result in an undesirably thick biofilm, which itself compromises membrane performance by reducing transmembrane water fluxes. To reduce the biofilm thickness when it is overly thick, we introduced a blue light-activated PDE gene into the *E. coli* strain (Ryu et al., 2017). The blue light-responsive c-di-GMP module consists of the engineered protein EB1, a bacteriophytochrome c-di-GMP hydrolase (Ryu et al., 2017). Following irradiation with blue light, the photoreceptor (BLUF) in EB1 undergoes conformational changes and activates PDE output domain (EAL), thereby degrading intracellular c-di-GMP to disperse biofilm (Figure 5.1).

Figure 5.1 Schematic illustration of the gene circuits in engineered *E. coli*. Dichromatic optogenetic module for regulating intracellular c-di-GMP levels comprising of a NIR light regulated DGC, BphS, and a blue light activated PDE, EB1. Quorum quenching gene circuit for producing AiiO, to reduce AHL regulated biofilm formation by other bacteria. A circle in front of each gene indicates a RBS; a T-sign at the end of the operon indicates a transcription terminator.

In addition, a gene encoding a QQ enzyme was introduced into the light-responsive *E. coli* strain. The QQ gene circuit in this study consists of the gene *aiiO* from *Ochrobactrum anthropi* (Oh et al., 2017). The AHL acylase AiiO produced by the light-responsive QQ *E. coli* strain would then break down AHL produced by other bacteria and thereby inhibit their biofilm formation. A gene encoding a variant of red fluorescent

protein, mCHERRY, was constitutively expressed in the QQ gene circuit to track the biofilm formation and dispersal on FO membranes by CLSM imaging (Figure 5.1).

5.3.2 Light-controllable development of the engineered *E. coli* biofilm

The DGC and PDE activities of BphS and EB1 in the light-responsive strain were assayed using c-di-GMP-dependent curly fimbriae formation (Hammar et al., 1995; Romling, 2005). Colonies of *E. coli*/pYYDT-RB, expressing the *bphS-bphO-eb1*, in the dark or with exposure to blue light were non-pigmented, which is indicative of low intracellular c-di-GMP levels (Figure 5.2A). However, colonies when grown under NIR light turned red due to the binding of Congo red to curly fimbriae, which is indicative of an elevated c-di-GMP level (Figure 5.2A). In contrast, the control strain, *i.e.*, *E. coli*/pYYDT harboring the empty vector, produced non-pigmented colonies under NIR light (Figure 5.2A).

Figure 5.2 The performance of the light-responsive gene circuit under NIR light and blue light. A) c-di-GMP-dependent curli fimbriae synthesis in *E. coli*. The control strain *E.*

coli/pYYDT and the light-responsive strain *E. coli*/pYYDT-RB were grown on Congo red plates for 24 h at 25 °C in the dark or when exposed to NIR light or blue light. (B) Total biofilm biomass (OD_{550nm}) of the control strain *E. coli*/pYYDT and the light-responsive strain *E. coli*/pYYDT-RB in each well after incubation for 24 h at 25 °C, without shaking, under dark, NIR light and blue light. (C) Intracellular c-di-GMP concentration (pmol/mg protein) in the light-responsive strain *E. coli*/pYYDT-RB after incubation for 24 h at 25°C in the dark or when exposed to NIR light or blue light. Experiments were conducted in triplicate. Error bar represents SD. ** $P < 0.005$ and *** $P < 0.0005$, Student's *t* test.

Biofilm formation assay for the light-responsive strain and the control strain was conducted in a 24-well microplate. For the light-responsive strain, biofilm biomass under NIR light was ~1.5-fold of the biofilm formed under dark, while the biofilm biomass under blue light was ~60% of the biofilm biomass incubated in the dark (Figure 5.2B). In contrast, no significant difference in the biofilm biomass was observed for the control strain under all conditions tested (Figure 5.2B). The intracellular c-di-GMP level of the *E. coli*/pYYDT-RB strain expressing *bphS-bphO-ebI* under NIR light was ~1.2-fold of that under dark condition (1.16 versus 0.96 pmol mg⁻¹ total protein) (Figure 5.2C).

To examine the effect of NIR light on biofilm formation in biofilm flow cells, we exposed a 24-h preformed light-responsive biofilm to NIR light and observed a ~20% increase in biofilm biovolume within 24 h. Following the NIR light irradiation, we exposed the biofilm to blue light and observed ~60% decrease in biofilm biovolume after 24 h. In

contrast, the preformed control biofilm showed no significant difference in biofilm biovolume over 24 h under NIR light or blue light (Figure 5.3).

Figure 5.3 Biofilm formation of the control *E. coli/pYYDT* and light-responsive *E. coli/pYYDT-RB* strains in biofilm flow cell. . (A) CLSM images of biofilms at 24, 72 and 120 h. The red line represents biofilms formed under NIR light and the blue line represents biofilms formed under blue light. Each CLSM image contains one plan view and two sectional views of the biofilm. Scale bar, 20 μm . (B) Biofilm biovolume (expressed as

biovolume per unit base area). The red and blue zones represent incubation under NIR light and blue light, respectively. Error bar represents the standard deviation.

To further examine the dynamics of biofilm formation on FO membranes under sequential exposure to NIR or blue light, biofilms of the light-responsive strain and the control strain were grown in an in-house-developed, CLSM-compatible membrane flow cell (Mukherjee et al., 2016) with commercially available thin-film composite FO membranes (Figure 5.4A). Under dark conditions, no significant difference was observed for the 48-h biofilms for the light-responsive or control strains in terms of biofilm biovolume ($46.9 \pm 4.8 \mu\text{m}^3 \mu\text{m}^{-2}$ versus $39.8 \pm 7.0 \mu\text{m}^3 \mu\text{m}^{-2}$) and biofilm thickness ($19.2 \pm 1.6 \mu\text{m}$ versus $16.2 \pm 2.3 \mu\text{m}$) (Figure 5.4C, D). After exposure to blue light for 24 h, the biovolume of the light-responsive biofilm decreased to $24.9 \pm 8.5 \mu\text{m}^3 \mu\text{m}^{-2}$, while the control biofilm biovolume increased to $60.2 \pm 9.7 \mu\text{m}^3 \mu\text{m}^{-2}$ at 72 h (Figure 5.4C). Subsequently, when the biofilms were exposed to NIR light (for 24 h) followed by another exposure to blue light (for 24 h), biovolume and thickness for the light-responsive biofilm gradually increased and then decreased to $12.9 \pm 3.9 \mu\text{m}^3 \mu\text{m}^{-2}$ and $4.9 \pm 1.2 \mu\text{m}$, respectively, while the control biofilm did not respond to the light exposure (Figure 5.4C and D).

In addition to biofilm dynamics on the FO membranes, we also observed that the transmembrane water flux for the FO membranes with the light-responsive biofilm gradually decreased and then increased under exposure to NIR light and blue light. This correlates with the increase in biofilm observed under NIR light and the reduction in biofilm when exposed to blue light. After the operation of the FO process for 120 h, the

water flux was 1.5 ± 0.04 LMH, which was $\sim 10\%$ lower than the initial water flux (1.7 ± 0.05 LMH). In contrast, the control biofilm resulted in a constant decline of the water flux by $\sim 40\%$ from 1.6 ± 0.09 LMH to 1.0 ± 0.04 LMH (Figure 5.4E).

Figure 5.4 Biofouling dynamics of the control, *E. coli*/pYYDT, and light-responsive, *E. coli*/pYYDT-RB, strains on FO membrane in membrane flow cell. (A) The CLSM-compatible membrane flow cell for nondestructive monitoring of biofouling dynamics on membranes. The device is made of three independent channels that are divided by a flat sheet membrane into two different channels. (B) CLSM images of biofilms grown on the FO membrane at four different timepoints ($t = 48, 72, 96$ and 120 h). The blue line represents biofilms formed after 24 h of incubation under blue light, and the red line represents biofilms formed after 24 h of incubation under NIR light. Each CLSM image contains one plan view and two sectional views of the biofilm. Scale bar, $20\ \mu\text{m}$. (C) Biofilm biovolume (expressed as biovolume per unit base area) and (D) biofilm thickness. (E) Comparisons of permeate flux through membranes at different time points. The gray, blue, and red zones represent incubation in the dark and under blue light and NIR light, respectively. Experiments were conducted in triplicate and five representative images were acquired and quantified for each replicate. Error bar represents SD. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, Student's t test.

Taken together, our results demonstrate that the biovolume and thickness of the light-responsive *E. coli* biofilm with the synthetic optogenetic c-di-GMP module could be modulated using light to limit its impact on the transmembrane water flux.

5.3.3 QQ activity of the engineered *E. coli* biofilm and inhibitory effect on biofilm formation by *P. stewartii*

The gene encoding AiiO from *O. anthropi* (*aioO*) (Oh et al., 2017) was introduced to the light-responsive *E. coli* strain to enable production of the QQ enzyme for mitigating biofouling caused by other bacteria (Figure 5.1). This strain was referred to as *E. coli*/(pYYDT-RB + pAiiO) or the QQ biofilm strain. Another strain, *E. coli*/(pYYDT-RB + pmAiiO), in which *aioO* was mutated so that the strain was not capable of producing the QQ enzyme, was used as a negative control. To examine the QQ activity in the QQ biofilm strain, 3OC6-HSL (final concentration, 1 μ M) was added to the cell suspension ($OD_{600nm} \sim 0.3$) and the cell-free conditioned medium (i.e., supernatant from the bacterial culture). Residual 3OC6-HSL was assayed with the *E. coli* JB525 biosensor (Andersen et al., 2001). There was no obvious degradation of the AHL signal by the negative control strain *E. coli*/(pYYDT-RB + pmAiiO) (Figure 5.5A). In contrast, complete degradation of the added 3OC6-HSL by the *E. coli*/(pYYDT-RB + pAiiO) cells was observed within 2 h (Figure 5.5A). Inactivation of 3OC6-HSL was also observed for the cell-free conditioned medium, although the activity was much lower than when the cells were present (Figure 5.5B). This result is consistent with previous studies where the QQ enzyme has been shown to be intracellularly localized and AHL inactivation in most cases was found to occur within bacterial cells (Czajkowski et al., 2011; Jafra et al., 2006). We further tested the QQ activity of the QQ biofilm strain under light conditions and found that the exposure to NIR light or blue light did not affect the QQ activity (Figure 5.5C).

Figure 5.5 AHL degradation is mediated by quorum quenching enzymatic activities. (A) Inactivation of exogenous 3OC6-HSL by the light-responsive QQ strain, *E. coli*/(pYYDT-RB + pAiiO), were assessed over 24 h at room temperature. Residual 3OC6-HSL was quantified using the *E. coli* JB525 bioassay at 0, 2, 6, 12 and 24 h post incubation. (B) Inactivation of exogenous 3OC6-HSL by *E. coli*/(pYYDT-RB + pAiiO) cells and the cell-free supernatant. (C) Inactivation of exogenous 3OC6-HSL by *E. coli*/(pYYDT-RB + pAiiO) cells incubated in the dark, under NIR light and blue light. (D) CLSM images of biofilm formation by YFP tagged *P. stewartii* on preformed light-responsive QQ biofilm *E. coli*/(pYYDT-RB + pAiiO) and QQ control biofilm *E. coli*/(pYYDT-RB + pmAiiO) on biofilm flow cells after 24 h. Scale bar, 20 μ m. Experiments were conducted in triplicate.

To demonstrate that the QQ biofilm strain can inhibit biofilm formation by other bacteria, biofilm formation of *P. stewartii* on a preformed QQ biofilm was investigated. *P. stewartii*, an isolate from wastewater, produces a wide range of AHL signals, and its biofilm formation is regulated by AHL-based QS signaling (Koutsoudis et al., 2006; Mohanty et al., 2016). Note that, unlike *P. stewartii*, the biofilm formation in *E. coli* was independent from the AHL signals (Sharma et al., 2016), and therefore, the QQ enzyme (AiiO) produced by the engineered *E. coli* strain has no interference with its own biofilm formation. Biofilms of the engineered QQ *E. coli* strain and the negative control, tagged with mCHERRY, were pre-grown on glass slides in biofilm flow cells for 24 h and then challenged with the YFP-tagged *P. stewartii*. The pre-formed biofilm producing the QQ enzyme inhibited the biofilm formation by *P. stewartii* while the QQ negative biofilm did not interfere with the biofilm formation by *P. stewartii* (Figure 5.5D). In addition, the negative control *E. coli* lacking QQ activity did not inhibit *P. stewartii* growth in biofilm cocultures (Figure 5.6). These results show that the engineered, light-responsive QQ *E. coli* strain is capable of effectively reducing biofilm formation by other bacteria.

Figure 5.6 Coculture biofilm formation by the mCHERRY-tagged control *E. coli* (QQ negative) and YFP-tagged *P. stewartii*. (A) CLSM image of co-culture biofilm at 24 h. Scale bar, 20 μm . (B) Biofilm biovolume (expressed as biovolume per unit base area). $P > 0.1$, Student's *t* test.

5.3.4 Light-modulated biofouling dynamics on FO membranes in membrane flow cells

We further tested the activity of the light-responsive QQ biofilm in FO membrane-based water purification processes using an in-housed built membrane flow cell under FO operation mode (Figure 5.4A). The membranes were first colonized with the light-responsive QQ strain *E. coli*/(pYYDT-RB + pAiiO) or the control strain *E. coli*/(pYYDT-RB + pmAiiO) (the light-responsive strain with no AiiO activity) for 24 h in FO operation mode. The preformed biofilms on the membranes were challenged with *P. stewartii*, and then the flow cells were allowed to run for 24 h in the dark. Biofilm formation by *P.*

stewartii on FO membranes without the preformed *E. coli* biofilms was also monitored for comparison.

After 24 h of colonization on the preformed light-responsive QQ biofilm, a small amount of *P. stewartii* biomass ($3.5 \pm 1.8 \mu\text{m}^3 \mu\text{m}^{-2}$) was observed (Figure 5.7A). In contrast, the membranes with a preformed control biofilm lacking QQ activity and without preformed biofilms were covered by *P. stewartii* with a biovolume of $17.4 \pm 4.9 \mu\text{m}^3 \mu\text{m}^{-2}$ and $33 \pm 5.6 \mu\text{m}^3 \mu\text{m}^{-2}$, respectively (Figure 5.7A). The results suggest that the light-responsive QQ biofilm was effective in inhibiting FO membrane biofouling caused by other bacteria. However, excessive biofilm formation by the light-responsive QQ *E. coli* strain could compromise membrane performance (e.g., reduction in transmembrane water flux). Therefore, we tested the use of NIR light and blue light to modulate the engineered light-responsive QQ biofilm growth and the biofouling dynamics on the FO membranes.

Figure 5.7 Biofouling dynamics of *P. stewartii* in the presence of the light-responsive QQ biofilm, *E. coli*/(pYYDT-RB + pAiiO), and a QQ control biofilm, *E. coli*/(pYYDT-RB +

pmAiiO), in a membrane flow cell. (A) CLSM images of biofilms formed on the FO membrane at 24, 48, 72 and 96 h. The blue line represents imaging after 24 h of incubation under blue light, and the red line represents imaging after 24 h of incubation under NIR light. Each CLSM image contains one plan view and two sectional views of the biofilm. Scale bar, 20 μm . (B) Total biofilm biovolume of the consortium (expressed as biovolume per unit base area). (C) Total biofilm thickness of the consortium. (D) Biofilm biovolume of *P. stewartii* in different consortium at 96 h. (E) Comparisons of permeate flux through membranes at different time points. The gray, blue, and red zones represent incubation in the dark or under blue light and NIR light, respectively. Error bar represents SD. * $P < 0.05$ and ** $P < 0.005$, *** $P < 0.0005$, Student's *t* test.

Preformed (from $t = 0$ to 24 h) membrane biofilms of the *P. stewartii* alone, the *P. stewartii* + *E. coli*/(pYYDT-RB + pAiiO) consortium, and the *P. stewartii* + *E. coli*/(pYYDT-RB + pmAiiO) consortium were subjected to sequential exposures to different lights: blue light for 24 h (from $t = 24$ to 48 h), then NIR light for 24 h ($t = 48$ to 72 h), and followed by blue light again for 24 h ($t = 72$ to 96 h). Before exposure to light ($t = 24$ h), biofilm biovolume and thickness for the three preformed biofilms were comparable (biovolume: $33 \pm 5.6 \mu\text{m}^3 \mu\text{m}^{-2}$ versus $43.8 \pm 7.1 \mu\text{m}^3 \mu\text{m}^{-2}$ versus $47.5 \pm 12 \mu\text{m}^3 \mu\text{m}^{-2}$; thickness: $10 \pm 1.9 \mu\text{m}$ versus $14.5 \pm 1.4 \mu\text{m}$ versus $12.9 \pm 4.4 \mu\text{m}$). In the presence of the preformed light-responsive QQ biofilm, total biofilm biovolume and thickness decreased to $24.3 \pm 7.8 \mu\text{m}^3 \mu\text{m}^{-2}$ and $9.2 \pm 3.0 \mu\text{m}$, respectively, after the first exposure to blue light for 24 h (at $t = 48$ h) (Figure 5.7B and C). The amount of biofilms was significantly lower than that formed in the presence of the control biofilm (39 ± 3.4

$\mu\text{m}^3 \mu\text{m}^{-2}$, $14.4 \pm 2.7 \mu\text{m}$) and the *P. stewartii* biofilm alone ($56.7 \pm 9.2 \mu\text{m}^3 \mu\text{m}^{-2}$; $19.8 \pm 2.8 \mu\text{m}$) (Figure 5.7B and C). Although the control biofilm formed by *E. coli*/(pYYDT-RB + pmAiiO) did not produce QQ enzyme, it could respond to the blue light. Hence, after 24 h of blue light exposure, the total biofilm biovolume formed in the presence of the preformed control biofilm was ~31% lower than the *P. stewartii* biofilm alone at $t = 48$ h (Figure 5.7B). Exposure of *E. coli*/(pYYDT-RB + pAiiO) biofilms to NIR light (for 24 h from $t = 48 - 72$ h) followed by blue light (for 24 h from $t = 72 - 96$ h) resulted in a light-mediated increase and then decrease in total biofilm biomass, reaching a biovolume of $18.7 \pm 3.4 \mu\text{m}^3 \mu\text{m}^{-2}$ at $t = 96$ h. In comparison, the total biofilm formed in the presence of the control biofilm that did not produce QQ enzyme exhibited a final biovolume of $37.4 \pm 12.5 \mu\text{m}^3 \mu\text{m}^{-2}$ at $t = 96$ h (Figure 5.7B). Although *P. stewartii* lacked the light-responsive module and did not respond to NIR light or blue light, a slightly lower biofilm biovolume at $t = 96$ h was observed for *P. stewartii* biofilm alone. This is likely due to the uncontrolled “dispersal” event that is often observed for a mature biofilm as a result of hydrodynamic sloughing and/or genetically programmed cell detachment triggered by local physicochemical conditions (Kaplan, 2010; O'Toole et al., 2000). In addition, although the control *E. coli* lacked QQ activity and a natural inhibition to *P. stewartii* growth, it seemed that the biofilm development of *P. stewartii* was inhibited by the presence of a preformed biofilm of the control *E. coli* (lacking QQ activity) (Figure 5.7A). It is important to note that surface properties including physicochemical characteristics such as hydrophobicity, roughness, and surface charges are expected to differ greatly between the FO membrane and the preformed *E. coli* biofilm. The difference in surface physiochemical properties would affect the biofilm development of *P. stewartii* (Palmer et al., 2007). Furthermore,

the control *E. coli* (lacking QQ activity) is light responsive, and the blue light-mediated dispersal would result in the displacement of the *P. stewartii* biofilm colonized on the surface of control *E. coli* biofilm.

The thickness of the biofilms formed in the presence of preformed light-responsive QQ biofilm exhibited a similar light-mediated increase and decrease trend, which was not observed for the biofilms formed in the presence of pre-formed control biofilm or the *P. stewartii* biofilm. The final thickness (at $t = 96$ h) of the biofilms formed in the presence of the preformed light-responsive QQ biofilm was $9.4 \pm 2.3 \mu\text{m}$, which is significantly lower than those for the biofilms formed in the presence of preformed control biofilm ($15.3 \pm 2.2 \mu\text{m}$) and the *P. stewartii* biofilm alone ($16.9 \pm 1.3 \mu\text{m}$) (Figure 5.7C). In addition, at $t = 96$ h, about $0.3 \pm 0.2 \mu\text{m}^3 \mu\text{m}^{-2}$ of *P. stewartii* was found on the membrane surfaces in the presence of the preformed light-responsive QQ biofilm, which is significantly lower than that with the preformed control biofilm ($15 \pm 5.1 \mu\text{m}^3 \mu\text{m}^{-2}$) (Figure 5.7D).

Throughout the course of the biofouling experiments, the transmembrane water flux for the membranes with the preformed control biofilm and the *P. stewartii* biofilm exhibited a decline of $\sim 12\%$ and $\sim 18\%$, respectively (Figure 5.7E). In contrast, in the presence of the light-responsive QQ biofilm, only about 6% reduction in the flux was observed (Figure 5.7E). Recent studies have reported that QQ significantly reduced the amount of EPS on membrane surfaces and triggered sloughing off of the biofilms, reducing biofouling (Kim et al., 2013; Oh et al., 2017). The higher flux achieved by the engineered light-responsive QQ biofilm correlated well with the QQ effect reported in other experimental systems (Kim et al., 2013; Oh et al., 2017). The transmembrane water flux

for the FO systems in the presence of a preformed light-responsive biofilm also showed light-mediated differences, suggesting that the effect of the preformed light-responsive QQ biofilm on the membrane performance can be effectively controlled by light.

5.4 Conclusion

We report the engineering of a light-responsive QQ biofilm by harnessing the power of QQ and c-di-GMP regulatory mechanisms in biofilms and demonstrate the potential application of the engineered biofilm in mitigating biofouling of water purification FO membranes. In this study, QQ activity was used to show the effectiveness of the light-responsive biofilm to limit its thickness while exhibiting desirable functions. The c-di-GMP-targeted optogenetic approach for controllable biofilm development we have demonstrated here should prove widely applicable for designing other thickness-controllable biofilm-enabled applications, for example, efficient biofilm-mediated biocatalysis for chemical synthesis. Our study exemplifies the potential for translational research from biofilm biology to biofilm engineering for applied and environmental biotechnology.

Chapter 6. Summary and Recommendations

6.1 Summary

Biofilms have been demonstrated as promising biocatalysts for applications in environmental and industrial biotechnologies. One of the most important factors influencing overall performance of biofilm-mediated biocatalysis is biofilm thickness. It is highly desirable to maintain the biofilm thickness within an optimal range which allows for efficient mass transfer and biocatalysis. Since c-di-GMP plays an important role in biofilm formation, regulating c-di-GMP levels serves as a suitable strategy for modulating biofilm development. In comparison to chemical inducers, optogenetic tools are ideal for controlling biofilms, as light is non-invasive, controllable and cost-efficient. Therefore, to engineer biofilms with a controllable thickness for efficient and robust biofilm-mediated bioprocesses, we genetically engineered a NIR light-responsive *S. oneidensis* biofilm for bioelectricity generation (Chapter 3), a NIR light-responsive *E. coli* biofilm for tryptophan production (Chapter 4) and a light-responsive, quorum quenching *E. coli* biofilm for biofouling control on water purification membrane (Chapter 5) by constructing optogenetic c-di-GMP gene circuits that respond to NIR light or dichroic light (NIR light and blue light). The main results and conclusions are summarized as follows:

- The constructed NIR light-responsive c-di-GMP module, which involves photoactivated synthesis of c-di-GMP catalyzed by an engineered bacteriophytochrome diguanylate cyclase (BphS), enhances the biofilm formation of *S. oneidensis* on anodes to increase electrical output by using NIR light to

increase c-di-GMP levels. This study demonstrated the feasibility of using light to modulate biofilm formation and improve the biofilm-based bioprocess.

- By introducing the NIR light-responsive c-di-GMP module and tryptophan synthase genes into *E. coli*, we engineer a NIR light-responsive, tryptophan-producing *E. coli* biofilm. NIR light significantly increases c-di-GMP level in *E. coli*. The high c-di-GMP concentration induces biofilm formation in submerged biofilm reactors, which results in higher bioconversion of indole to tryptophan in a certain range of biofilm thickness.

A constantly high c-di-GMP level causes an overly thick biofilm of *E. coli*, leading to high mass transfer limitations and a high amount of unproductive biomass at the bottom of the biofilm. Consequently, these result in low specific catalytic activity and decreased tryptophan concentration in the effluent.

- To maintain the biofilm thickness in an optimal range for efficient and robust biofilm-based bioprocesses, the bidirectional control of c-di-GMP levels are achieved through a dichromatic, optogenetic c-di-GMP gene circuit in which the bacterial cells sense NIR light and blue light to adjust its biofilm formation by regulating the c-di-GMP level.
- A biofilm of *E. coli*, which is engineered by introducing the dichromatic, optogenetic c-di-GMP and quorum quenching gene circuits, controls its own biofilm formation through the modulation of intracellular c-di-GMP concentration by using NIR light or blue light and inhibits biofouling caused by other bacteria via production of QQ enzyme. This study demonstrated the potential application of the

engineered light-responsive QQ biofilm in mitigating biofouling of water purification membranes.

6.2 Recommendations for future research

The use of optogenetic c-di-GMP gene circuits engineered the light-responsive biofilms through adjusting c-di-GMP levels in cells by using light. In our studies, we found the long response time of biofilm thickness to NIR light. It might be because of the low activity of the c-di-GMP synthase or the interference of the native c-di-GMP signaling networks. For precise control of the biofilm thickness, the future studies will focus on the optimization of the c-di-GMP synthase and hydrolase and the construction of bacterial chassis with a low native c-di-GMP level.

6.2.1 Optimize the activity of the c-di-GMP synthase and hydrolase in the light-responsive gene circuit

Although the biofilm thickness of *E. coli*/pYYDT-RB strain expressing *bphS-bphO-ebf* can be modulated through adjusting its c-di-GMP level by NIR light and blue light in Chapter 5, the intracellular c-di-GMP level and biofilm biomass of the light-responsive strain show a relatively low fold changes under NIR light as compared to under dark (Figure 5.2). Moreover, we observed only a ~20% increase in the biofilm biovolume of the light-responsive strain when exposed to NIR light for 24 h (Figure 5.3), suggesting a long response time of engineered biofilm to NIR light. A plausible reason may be due to the c-di-GMP hydrolase (EB1) exhibiting a residual PDE activity even in the absence of blue light, which could have compromise the NIR light affect to elevate the c-di-GMP level.

This issue may be addressed in further studies using two ways: (i) the directed evolution of BphS for enhancing its activity in the NIR light and/or (ii) the directed evolution of EB1 for reducing its activity in the absence of blue light. Therefore, directed evolution of BphS and EB1 using error-prone polymerase chain reaction (PCR) are to be conducted as future studies.

6.2.2 Construct bacterial chassis with a low native c-di-GMP level for the improvement of the c-di-GMP gene circuit performance

An important factor affecting the c-di-GMP gene circuit performance in hosts is the native c-di-GMP levels that are regulated by the endogenous c-di-GMP synthases and hydrolases. The native c-di-GMP signaling networks in hosts may compromise the effect of exogenous gene circuits on the resulting c-di-GMP levels. In previous work, to improve the photodynamic range of NIR light-responsive c-di-GMP module, the c-di-GMP level contributed by native *E. coli* DGCs was overpowered through the overexpression of YhjH, a PDE from *E. coli* (Ryu and Gomelsky, 2014). The expression level of PDE is of great importance for the overall performance of c-di-GMP gene circuits. A low expression level of PDE is insufficient to reduce the background c-di-GMP level, whereas an overly high expression level may override the activity of exogenous DGCs in gene circuits. Although previous study obtained an optimal expression level by using a series of ribosome-binding sites (RBSs) with different strengths to adjust the expression level of PDE (Ryu and Gomelsky, 2014), such approach is timing-consuming and may not be suitable for broad application in different gene circuits and hosts. It is because any perturbations of DGC activity in gene circuits and native c-di-GMP level in different hosts require the tuning of

PDE expression level. Therefore, it is desirable for a host itself to possess a low background level of c-di-GMP to allow an improvement to the introduced c-di-GMP gene circuit performance. To obtain such a bacterial chassis, it is aimed to deplete a host's native c-di-GMP pools by downregulating the gene expression of native DGCs. With the rapid development of synthetic biology, genome-editing approaches, such as (CRISPR)/CRISPRi and Hfq-dependent sRNA, provide facilitation to achieve this genome-scale manipulation.

Since *E. coli* is one of the model organisms in biofilm studies, one of our future studies will focus on the construction of a *E. coli* BL21(DE3) chassis with a low native c-di-GMP level. There are 11 genes encoding DGCs in the genome of *E. coli* BL21(DE3). To verify the prevalent DGCs, we will design Hfq-dependent sRNA to downregulate each DGC genes and to test the resulting c-di-GMP levels using Congo red agar plates based on the c-di-GMP-dependent curly fimbriae formation. By downregulating one or several native DGC genes which are prevalent for c-di-GMP synthesis, we could obtain a *E. coli* BL21(DE3) chassis in which the c-di-GMP level is tightly controlled by constructed c-di-GMP gene circuits.

6.2.3 Engineer a biofilm with a self-regulated c-di-GMP level.

Although the use of light, rather than chemicals, to control biofilm development is highly desirable, the major concern of using light to control biofilm formation is the limitation of light penetration in some of reactors, such as the hollow fiber bioreactors. Therefore, self-regulated biofilm would be more desirable. In living cells, physiological processes, such as cardiac function, insulin secretion, and respiration, are governed by

synchronized oscillators as a result of a vast range of intercellular coupling mechanisms (Danino et al., 2010). One defining aim of synthetic biology is to engineer gene circuits with predictive functionality in living cells. In the last decade, autonomous and self-sustained oscillator circuits have attracted significant attention because of dynamical regulation and applications in controlling repair, signaling and metabolic pathways (Fussenegger, 2010; McMillen et al., 2002; Singh, 2014). Hence, another future study is the construction of self-regulated biofilms by creating a c-di-GMP oscillator that dynamically controls intracellular c-di-GMP level. For the precise self-regulation of c-di-GMP levels, real-time detection of c-di-GMP levels is of importance. Although c-di-GMP levels can be determined using liquid chromatography-mass spectrometry, it is highly challenging to achieve real-time detection of c-di-GMP levels *in vivo*. Therefore, constructing a sense system in response to c-di-GMP levels is of great significance for the achievement of c-di-GMP oscillator.

To gauge c-di-GMP levels *in vivo*, Rybtke et al. created a fluorescence-based reporter by transcriptionally fusing the c-di-GMP-responsive promoter, P_{cdrA} , to genes green fluorescent protein-encoding gene, *gfp* (Rybtke et al., 2012). However, this reporter is limited in *P. aeruginosa* as the strength of c-di-GMP responsive promoter P_{cdrA} is extremely low in non-*Pseudomonas* strains. Therefore, one of important work for the achievement of c-di-GMP oscillator is to construct a broadly applied biosensor that is readily adaptable for gauging c-di-GMP levels in different bacteria. We will further use this responsive system to construct the c-di-GMP oscillator in a suitable organism.

References

- Achilli, A., Cath, T.Y., Marchand, E.A., Childress, A.E., 2009. The forward osmosis membrane bioreactor: A low fouling alternative to MBR processes. **Desalination** 239(1), 10-21.
- Alhede, M., Bjarnsholt, T., Jensen, P.Ø., Phipps, R.K., Moser, C., Christophersen, L., Christensen, L.D., van Gennip, M., Parsek, M., Høiby, N., Rasmussen, T.B., Givskov, M., 2009. *Pseudomonas aeruginosa* recognizes and responds aggressively to the presence of polymorphonuclear leukocytes. **Microbiology** 155(11), 3500-3508.
- Allesen-Holm, M., Barken, K.B., Yang, L., Klausen, M., Webb, J.S., Kjelleberg, S., Molin, S., Givskov, M., Tolker-Nielsen, T., 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. **Molecular Microbiology** 59(4), 1114-1128.
- Amikam, D., Galperin, M.Y., 2006. PilZ domain is part of the bacterial c-di-GMP binding protein. **Bioinformatics** 22(1), 3-6.
- Miller M.B., Bassler, B.L., 2001. Quorum Sensing in Bacteria. **Annual Review of Microbiology** 55(1), 165-199.
- Andersen, J.B., Heydorn, A., Hentzer, M., Eberl, L., Geisenberger, O., Christensen, B.B., Molin, S., Givskov, M., 2001. gfp-based N-acyl homoserine-lactone sensor systems for detection of bacterial communication. **Applied and Environmental Microbiology** 67(2), 575-585.
- Benedetti, I., de Lorenzo, V., Nikel, P.I., 2016. Genetic programming of catalytic *Pseudomonas putida* biofilms for boosting biodegradation of haloalkanes. **Metabolic Engineering** 33, 109-118.
- Bishop, P.L., Zhang, T.C., Fu, Y.-C., 1995. Effects of biofilm structure, microbial distributions and mass transport on biodegradation processes. *Water Science and Technology* 31(1), 143-152.
- Blenkinsopp, S.A., Costerton, J.W., 1991. Understanding bacterial biofilms. **Trends in Biotechnology** 9(1), 138-143.

- Borlee, B.R., Goldman, A.D., Murakami, K., Samudrala, R., Wozniak, D.J., Parsek, M.R., 2010. *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. **Molecular Microbiology** 75(4), 827-842.
- Borole, A.P., Reguera, G., Ringeisen, B., Wang, Z.-W., Feng, Y., Kim, B.H., 2011. Electroactive biofilms: Current status and future research needs. **Energy & Environmental Science** 4(12), 4813-4834.
- Botyanszki, Z., Tay, P.K.R., Nguyen, P.Q., Nussbaumer, M.G., Joshi, N.S., 2015. Engineered catalytic biofilms: Site-specific enzyme immobilization onto *E. coli* curli nanofibers. **Biotechnology and Bioengineering** 112(10), 2016-2024.
- Boyd, C.D., O'Toole, G.A., 2012. Second messenger regulation of biofilm formation: Breakthroughs in understanding c-di-GMP effector systems. **Annual Review of Cell and Developmental Biology** 28, 439-462.
- Branda, S.S., Vik, S., Friedman, L., Kolter, R., 2005. Biofilms: the matrix revisited. **Trends in Microbiology** 13(1), 20-26.
- Brenner, K., Karig, D.K., Weiss, R., Arnold, F.H., 2007. Engineered bidirectional communication mediates a consensus in a microbial biofilm consortium. **Proceedings of the National Academy of Sciences of the United States of America** 104(44), 17300-17304.
- Bretschger, O., Obraztsova, A., Sturm, C.A., Chang, I.S., Gorby, Y.A., Reed, S.B., Culley, D.E., Reardon, C.L., Barua, S., Romine, M.F., Zhou, J., Beliaev, A.S., Bouhenni, R., Saffarini, D., Mansfeld, F., Kim, B.-H., Fredrickson, J.K., Nealson, K.H., 2007. Current production and metal oxide reduction by *Shewanella oneidensis* MR-1 wild type and mutants. **Applied and Environmental Microbiology** 73(21), 7003-7012.
- Brooun, A., Liu, S., Lewis, K., 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. **Antimicrobial Agents and Chemotherapy** 44(3), 640-646.
- Cao, B., Ahmed, B., Kennedy, D.W., Wang, Z., Shi, L., Marshall, M.J., Fredrickson, J.K., Isern, N.G., Majors, P.D., Beyenal, H., 2011a. Contribution of extracellular polymeric

substances from *Shewanella* sp. HRCR-1 biofilms to U(VI) immobilization. **Environmental Science & Technology** 45(13), 5483-5490.

Cao, B., Majors, P.D., Ahmed, B., Renslow, R.S., Silvia, C.P., Shi, L., Kjelleberg, S., Fredrickson, J.K., Beyenal, H., 2012. Biofilm shows spatially stratified metabolic responses to contaminant exposure. **Environmental Microbiology** 14(11), 2901-2910.

Cao, B., Shi, L., Brown, R.N., Xiong, Y., Fredrickson, J.K., Romine, M.F., Marshall, M.J., Lipton, M.S., Beyenal, H., 2011b. Extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms: Characterization by infrared spectroscopy and proteomics. **Environmental Microbiology** 13(4), 1018-1031.

Chaudhuri, S.K., Lovley, D.R., 2003. Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. **Nature Biotechnology** 21(10), 1229-1232.

Chen, C.-Y., Nace, G.W., Irwin, P.L., 2003. A 6×6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. **Journal of Microbiological Methods** 55(2), 475-479.

Chua, S.L., Sivakumar, K., Rybtke, M., Yuan, M., Andersen, J.B., Nielsen, T.E., Givskov, M., Tolker-Nielsen, T., Cao, B., Kjelleberg, S., Yang, L., 2015. C-di-GMP regulates *Pseudomonas aeruginosa* stress response to tellurite during both planktonic and biofilm modes of growth. **Scientific Reports** 5, 10052.

Cologgi, D.L., Speers, A.M., Bullard, B.A., Kelly, S.D., Reguera, G., 2014. Enhanced uranium immobilization and reduction by *Geobacter sulfurreducens* biofilms. **Applied and Environmental Microbiology** 80(21), 6638-6646.

Colvin, K.M., Gordon, V.D., Murakami, K., Borlee, B.R., Wozniak, D.J., Wong, G.C., Parsek, M.R., 2011. The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. **PLoS Pathogens** 7(1), e1001264.

Colvin, K.M., Irie, Y., Tart, C.S., Urbano, R., Whitney, J.C., Ryder, C., Howell, P.L., Wozniak, D.J., Parsek, M.R., 2012. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. **Environmental Microbiology** 14(8), 1913-1928.

- Costerton, J.W., Lewandowski, Z., DeBeer, D., Caldwell, D., Korber, D., James, G., 1994. Biofilms, the customized microniche. **Journal of Bacteriology** 176(8), 2137.
- Cotter, P.A., Stibitz, S., 2007. c-di-GMP-mediated regulation of virulence and biofilm formation. **Current Opinion in Microbiology** 10(1), 17-23.
- Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., Penadés, J.R., 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. **Journal of Bacteriology** 183(9), 2888-2896.
- Czajkowski, R., Krzyzanowska, D., Karczewska, J., Atkinson, S., Przysowa, J., Lojkowska, E., Williams, P., Jafra, S., 2011. Inactivation of AHLs by *Ochrobactrum* sp. A44 depends on the activity of a novel class of AHL acylase. **Environmental Microbiology Reports** 3(1), 59-68.
- Danino, T., Mondragón-Palomino, O., Tsimring, L., Hasty, J., 2010. A synchronized quorum of genetic clocks. **Nature** 463(7279), 326-330.
- Davies, D.G., Parsek, M.R., Pearson, J.P., Igleski, B.H., Costerton, J.W., Greenberg, E.P., 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. **Science** 280(5361), 295-298.
- De Beer, D., Stoodley, P., Roe, F., Lewandowski, Z., 1994. Effects of biofilm structures on oxygen distribution and mass transport. **Biotechnology and Bioengineering** 43(11), 1131-1138.
- De Carvalho, C.C., 2007. Biofilms: recent developments on an old battle. **Recent Patents on Biotechnology** 1(1), 49-57.
- Dehio, C., Meyer, M., 1997. Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. **Journal of Bacteriology** 179(2), 538-540.
- Demirci, A., Cotton, J.C., Pometto, A.L., Harkins, K.R., Hinz, P.N., 2003. Resistance of *Lactobacillus casei* in plastic-composite-support biofilm reactors during liquid membrane extraction and optimization of the lactic acid extraction system. **Biotechnology and Bioengineering** 83(7), 749-759.

- Demirjian, D.C., Morís-Varas, F., Cassidy, C.S., 2001. Enzymes from extremophiles. **Current Opinion in Chemical Biology** 5(2), 144-151.
- Di Martino, P., Fursy, R., Bret, L., Sundararaju, B., Phillips, R.S., 2003. Indole can act as an extracellular signal to regulate biofilm formation of *Escherichia coli* and other indole-producing bacteria. **Canadian Journal of Microbiology** 49(7), 443-449.
- Ding, Y., Peng, N., Du, Y., Ji, L., Cao, B., 2014. Disruption of putrescine biosynthesis in *Shewanella oneidensis* enhances biofilm cohesiveness and performance in Cr(VI) immobilization. **Applied and Environmental Microbiology** 80(4), 1498-1506.
- Domka, J., Lee, J., Wood, T.K., 2006. YliH (BssR) and YceP (BssS) regulate *Escherichia coli* K-12 biofilm formation by influencing cell signaling. **Applied and Environmental Microbiology** 72(4), 2449-2459.
- Du, Z., Li, H., Gu, T., 2007. A state of the art review on microbial fuel cells: a promising technology for wastewater treatment and bioenergy. **Biotechnology Advances** 25(5), 464-482.
- Eickhoff, M.J., Bassler, B.L., 2018. SnapShot: Bacterial quorum sensing. **Cell** 174(5), 1328-1328.e1.
- Erable, B., Duțeanu, N.M., Ghangrekar, M.M., Dumas, C., Scott, K., 2010. Application of electro-active biofilms. **Biofouling** 26(1), 57-71.
- Fang, X., Gomelsky, M., 2010. A post-translational, c-di-GMP-dependent mechanism regulating flagellar motility. **Molecular Microbiology** 76(5), 1295-1305.
- Fenno, L., Yizhar, O., Deisseroth, K., 2011. The development and application of optogenetics. **Annual Review of Neuroscience** 34(1), 389-412.
- Fernandez-Rodriguez, J., Moser, F., Song, M., Voigt, C.A., 2017. Engineering RGB color vision into *Escherichia coli*. **Nature Chemical Biology** 13(7), 706–708.
- Fetzner, S., 2015. Quorum quenching enzymes. **Journal of Biotechnology** 201, 2-14.
- Flemming, H.C., Neu, T.R., Wozniak, D.J., 2007. The EPS matrix: The “house of biofilm cells”. **Journal of Bacteriology** 189(22), 7945-7947.

- Flemming, H.C., Wingender, J., 2010. The biofilm matrix. **Nature Reviews Microbiology** 8(9), 623-633.
- Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. **Nature Reviews Microbiology** 14(9), 563-575.
- Flemming, H.C., Schaule, G., Griebel, T., Schmitt, J., Tamachkierowa, A., 1997. Biofouling—the Achilles heel of membrane processes. **Desalination** 113(2), 215-225.
- Frank, A.E., Malvankar, N., Nevin, K.P., 2014. Bacterial biofilms: the powerhouse of a microbial fuel cell. **Biofuels** 4(12), 4813-4834.
- Friedman, L., Kolter, R., 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. **Molecular Microbiology** 51(3), 675-690.
- Fu, P., 2006. A perspective of synthetic biology: Assembling building blocks for novel functions. **Biotechnology Journal** 1(6), 690-699.
- Fussenegger, M., 2010. Synthetic biology: Synchronized bacterial clocks. *Nature* 463, 301-302.
- Ghafoor, A., Hay, I.D., Rehm, B.H.A., 2011. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. **Applied and Environmental Microbiology** 77(15), 5238-5246.
- Glansdorff, F.G., Thomas, G.L., Lee, J.K., Dutton, J.M., Salmond, G.P.C., Welch, M., Spring, D.R., 2004. Synthesis and stability of small molecule probes for *Pseudomonas aeruginosa* quorum sensing modulation. **Organic & Biomolecular Chemistry** 2(22), 3329-3336.
- Gonzalez Barrios, A.F., Zuo, R., Hashimoto, Y., Yang, L., Bentley, W.E., Wood, T.K., 2006. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). **Journal of Bacteriology** 188(1), 305-316.

- Gross, R., Buehler, K., Schmid, A., 2013. Engineered catalytic biofilms for continuous large scale production of n-octanol and (S)-styrene oxide. **Biotechnology and Bioengineering** 110(2), 424-436.
- Gross, R., Hauer, B., Otto, K., Schmid, A., 2007. Microbial biofilms: New catalysts for maximizing productivity of long-term biotransformations. **Biotechnology and Bioengineering** 98(6), 1123-1134.
- Gross, R., Lang, K., Bühler, K., Schmid, A., 2010. Characterization of a biofilm membrane reactor and its prospects for fine chemical synthesis. **Biotechnology and Bioengineering** 105(4), 705-717.
- Gu, Y., Huang, J., Zeng, G., Shi, Y., Hu, Y., Tang, B., Zhou, J., Xu, W., Shi, L., 2018. Quorum quenching activity of indigenous quorum quenching bacteria and its potential application in mitigation of membrane biofouling. **Journal of Chemical Technology & Biotechnology** 93(5), 1394-1400.
- Guillen, C., Forestier, C., Balestrino, D., 2017. Biofilm dispersal: multiple elaborate strategies for dissemination of bacteria with unique properties. *Molecular microbiology* 105(2), 188-210.
- Gupta, K., Liao, J., Petrova, O.E., Cherny, K.E., Sauer, K., 2014. Elevated levels of the second messenger c-di-GMP contribute to antimicrobial resistance of *Pseudomonas aeruginosa*. **Molecular Microbiology** 92(3), 488-506.
- Halan, B., Buehler, K., Schmid, A., 2012. Biofilms as living catalysts in continuous chemical syntheses. **Trends in Biotechnology** 30(9), 453-465.
- Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. **Nature Reviews Microbiology** 2(2), 95-108.
- Hammar, M., Arnqvist, A., Bian, Z., Olsen, A., Normark, S., 1995. Expression of two csg operons is required for production of fibronectin- and congo red-binding curli polymers in *Escherichia coli* K-12. **Molecular Microbiology** 18(4), 661-670.
- Harrison, J.J., Ceri, H., Turner, R.J., 2007. Multimetal resistance and tolerance in microbial biofilms. **Nature Reviews Microbiology** 5(12), 928-938.

- Hatt, J., Rather, P., 2008. Role of bacterial biofilms in urinary tract infections, **Bacterial Biofilms** 322, 163-192.
- Hawver, L.A., Jung, S.A., Ng, W.L., 2016. Specificity and complexity in bacterial quorum-sensing systems. **FEMS Microbiology Reviews** 40(5), 738-752.
- Hengge, R., 2009. Principles of c-di-GMP signalling in bacteria. **Nature Reviews Microbiology** 7(4), 263-273.
- Henriques, I.D.S., Love, N.G., 2007. The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins. **Water Research** 41(18), 4177-4185.
- Herrero, M., de Lorenzo, V., Timmis, K.N., 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. **Journal of Bacteriology** 172(11), 6557-6567.
- Heydorn, A., Ersboll, B.K., Hentzer, M., Parsek, M.R., Givskov, M., Molin, S., 2000. Experimental reproducibility in flow-chamber biofilms. **Microbiology** 146 (10), 2409-2415.
- Hickman, J.W., Harwood, C.S., 2008. Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. **Molecular Microbiology** 69(2), 376-389.
- Hong, S.H., Hegde, M., Kim, J., Wang, X., Jayaraman, A., Wood, T.K., 2012. Synthetic quorum-sensing circuit to control consortial biofilm formation and dispersal in a microfluidic device. **Nature Communications** 3, 613.
- Hu, Y., Wu, Y., Mukherjee, M., Cao, B., 2017. A near-infrared light responsive c-di-GMP module-based AND logic gate in *Shewanella oneidensis*. **Chemical Communications** 53(10), 1646-1648.
- Hu, Y., Yang, Y., Katz, E., Song, H., 2015. Programming the quorum sensing-based AND gate in *Shewanella oneidensis* for logic gated-microbial fuel cells. **Chemical Communications** 51(20), 4184-4187.

- Huang, Y., Xia, A., Yang, G., Jin, F., 2018. Bioprinting living biofilms through optogenetic manipulation. **ACS Synthetic Biology** 7(5), 1195-1200.
- Jafra, S., Przysowa, J., Czajkowski, R., Michta, A., Garbeva, P., van der Wolf, J.M., 2006. Detection and characterization of bacteria from the potato rhizosphere degrading N-acyl-homoserine lactone. **Canadian Journal of Microbiology** 52(10), 1006-1015.
- Jenal, U., Reinders, A., Lori, C., 2017. Cyclic di-GMP: second messenger extraordinaire. **Nature Reviews Microbiology** 15(5), 271-284.
- Jiang, L., Song, X., Li, Y., Xu, Q., Pu, J., Huang, H., Zhong, C., 2018. Programming integrative extracellular and intracellular biocatalysis for rapid, robust, and recyclable synthesis of trehalose. **ACS Catalysis** 8(3), 1837-1842.
- Kaplan, J.B., 2010. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. **Journal of Dental Research** 89(3), 205-218.
- Kaplan, J.B., Ragunath, C., Ramasubbu, N., Fine, D.H., 2003. Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous β -hexosaminidase activity. **Journal of Bacteriology** 185(16), 4693-4698.
- Kargi, F., Eker, S., 2005. Removal of 2,4-dichlorophenol and toxicity from synthetic wastewater in a rotating perforated tube biofilm reactor. **Process Biochemistry** 40(6), 2105-2111.
- Kawasaki, H., Bauerle, R., Zon, G., Ahmed, S.A., Miles, E.W., 1987. Site-specific mutagenesis of the alpha subunit of tryptophan synthase from *Salmonella typhimurium*. Changing arginine 179 to leucine alters the reciprocal transmission of substrate-induced conformational changes between the alpha and beta 2 subunits. **Journal of Biological Chemistry** 262(22), 10678-10683.
- Kennedy, M.J., Hughes, R.M., Peteya, L.A., Schwartz, J.W., Ehlers, M.D., Tucker, C.L., 2010. Rapid blue-light-mediated induction of protein interactions in living cells. **Nature Methods** 7(12), 973-975.
- Kim, C.M., Kim, S.-J., Kim, L.H., Shin, M.S., Yu, H.W., Kim, I.S., 2014. Effects of phosphate limitation in feed water on biofouling in forward osmosis (FO) process. **Desalination** 349, 51-59.

- Kim, H.W., Oh, H.S., Kim, S.R., Lee, K.B., Yeon, K.M., Lee, C.H., Kim, S., Lee, J.K., 2013. Microbial population dynamics and proteomics in membrane bioreactors with enzymatic quorum quenching. **Applied Microbiology and Biotechnology** 97(10), 4665-4675.
- Kim, S.R., Oh, H.S., Jo, S.-J., Yeon, K.-M., Lee, C.H., Lim, D.J., Lee, C.H., Lee, J.K., 2013. Biofouling control with bead-entrapped quorum quenching bacteria in membrane bioreactors: Physical and biological effects. **Environmental Science & Technology** 47(2), 836-842.
- Komlenic, R., 2010. Rethinking the causes of membrane biofouling. **Filtration & Separation** 47(5), 26-28.
- Korber, D.R., Lawrence, J.R., Hendry, M.J., Caldwell, D.E., 1993. Analysis of spatial variability within mot⁺ and mot⁻ *Pseudomonas fluorescens* biofilms using representative elements. **Biofouling** 7(4), 339-358.
- Koutsoudis, M.D., Tsaltas, D., Minogue, T.D., von Bodman, S.B., 2006. Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. **Proceedings of the National Academy of Sciences of the United States of America** 103(15), 5983-5988.
- Kuchma, S.L., Ballok, A.E., Merritt, J.H., Hammond, J.H., Lu, W., Rabinowitz, J.D., O'Toole, G.A., 2010. Cyclic-di-GMP-mediated repression of swarming motility by *Pseudomonas aeruginosa*: the pilY1 gene and its impact on surface-associated behaviors. **Journal of Bacteriology** 192(12), 2950-2964.
- Kuchma, S.L., Brothers, K.M., Merritt, J.H., Liberati, N.T., Ausubel, F.M., O'Toole, G.A., 2007. BifA, a cyclic-Di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. **Journal of Bacteriology** 189(22), 8165-8178.
- Labbate, M., Queck, S.Y., Koh, K.S., Rice, S.A., Givskov, M., Kjelleberg, S., 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. **Journal of Bacteriology** 186(3), 692-698.

- Lasa, I., Penadés, J.R., 2006. Bap: A family of surface proteins involved in biofilm formation. **Research in Microbiology** 157(2), 99-107.
- Lee, J., Jayaraman, A., Wood, T.K., 2007. Indole is an inter-species biofilm signal mediated by SdiA. **BMC microbiology** 7(1), 42.
- Lee, J., Zhang, X.-S., Hegde, M., Bentley, W.E., Jayaraman, A., Wood, T.K., 2008. Indole cell signaling occurs primarily at low temperatures in *Escherichia coli*. **The Isme Journal** 2(10), 1007.
- Lee, S., Boo, C., Elimelech, M., Hong, S., 2010. Comparison of fouling behavior in forward osmosis (FO) and reverse osmosis (RO). **Journal of Membrane Science** 365(1), 34-39.
- Lendenmann, U., Spain, J.C., Smets, B.F., 1998. Simultaneous biodegradation of 2,4-dinitrotoluene and 2,6-dinitrotoluene in an aerobic fluidized-bed biofilm reactor. **Environmental Science & Technology** 32(1), 82-87.
- Levskaya, A., Chevalier, A.A., Tabor, J.J., Simpson, Z.B., Lavery, L.A., Levy, M., Davidson, E.A., Scouras, A., Ellington, A.D., Marcotte, E.M., Voigt, C.A., 2005. Engineering *Escherichia coli* to see light. **Nature** 438(7076), 441-442.
- Levskaya, A., Weiner, O.D., Lim, W.A., Voigt, C.A., 2009. Spatiotemporal control of cell signalling using a light-switchable protein interaction. **Nature** 461(7277), 997-1001.
- Li, X.Z., Hauer, B., Rosche, B., 2013. Catalytic biofilms on structured packing for the production of glycolic acid. **Journal of Microbiology and Biotechnology** 23(2), 195-204.
- Li, Z., Rosenbaum, M.A., Venkataraman, A., Tam, T.K., Katz, E., Angenent, L.T., 2011. Bacteria-based AND logic gate: a decision-making and self-powered biosensor. **Chemical Communications** 47(11), 3060-3062.
- Liang, Z.X., 2015. The expanding roles of c-di-GMP in the biosynthesis of exopolysaccharides and secondary metabolites. **Natural Product Reports** 32(5), 663-683.
- Lienhardt, J., Schripsema, J., Qureshi, N., Blaschek, H.P., 2002. Butanol production by *Clostridium beijerinckii* BA101 in an immobilized cell biofilm reactor. **Applied Biochemistry and Biotechnology** 98(1-9), 591-598.

- Liu, T., Yu, Y.Y., Deng, X.P., Ng, C.K., Cao, B., Wang, J.Y., Rice, S.A., Kjelleberg, S., Song, H., 2015. Enhanced *Shewanella* biofilm promotes bioelectricity generation. **Biotechnology and Bioengineering** 112(10), 2051-2059.
- Ma, L., Conover, M., Lu, H., Parsek, M.R., Bayles, K., Wozniak, D.J., 2009. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. **PLoS Pathogens** 5(3), e1000354.
- Mah, T.F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. **Trends in Microbiology** 9(1), 34-39.
- McDougald, D., Rice, S.A., Barraud, N., Steinberg, P.D., Kjelleberg, S., 2011. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. **Nature Reviews Microbiology** 10(1), 39-50.
- McMillen, D., Kopell, N., Hasty, J., Collins, J.J., 2002. Synchronizing genetic relaxation oscillators by intercell signaling. **Proceedings of the National Academy of Sciences of the United States of America** 99(2), 679-684.
- Merritt, J.H., Ha, D.-G., Cowles, K.N., Lu, W., Morales, D.K., Rabinowitz, J., Gitai, Z., O'Toole, G.A., 2010. Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. **mBio** 1(4), e00183-00110.
- Miller, E.M., Nickoloff, J.A., 1995. *Escherichia coli* electrotransformation. **Electroporation Protocols for Microorganisms** 47, 105-113.
- Min, B., Logan, B.E., 2004. Continuous electricity generation from domestic wastewater and organic substrates in a flat plate microbial fuel cell. **Environmental Science & Technology** 38(21), 5809-5814.
- Mohanty, A., Tan, C.H., Cao, B., 2016. Impacts of nanomaterials on bacterial quorum sensing: differential effects on different signals. **Environmental Science: Nano** 3(2), 351-356.
- Mohanty, A., Wei, L., Lu, L., Chen, Y., Cao, B., 2015. Impact of sublethal levels of single-wall carbon nanotubes on pyoverdine production in *Pseudomonas aeruginosa* and its environmental implications. **Environmental Science & Technology Letters** 2(4), 105-111.

- Montanaro, L., Poggi, A., Visai, L., Ravaioli, S., Campoccia, D., Speziale, P., Arciola, C.R., 2011. Extracellular DNA in biofilms. **The International Journal of Artificial Organs** 34(9), 824-831.
- Mukherjee, M., Hu, Y., Tan, C.H., Rice, S.A., Cao, B., 2018. Engineering a light-responsive, quorum quenching biofilm to mitigate biofouling on water purification membranes. **Science Advances** 4(12), eaau1459.
- Mukherjee, M., Menon, N.V., Liu, X., Kang, Y., Cao, B., 2016. Confocal laser scanning microscopy-compatible microfluidic membrane flow cell as a nondestructive tool for studying biofouling dynamics on forward osmosis membranes. **Environmental Science & Technology Letters** 3(8), 303-309.
- Müller, K., Weber, W., 2013. Optogenetic tools for mammalian systems. **Molecular Biosystems** 9(4), 596-608.
- Myers, C.R., Nealson, K.H., 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. **Science** 240(4857), 1319-1321.
- Nicolella, C., van Loosdrecht, M.C.M., Heijnen, J.J., 2000. Wastewater treatment with particulate biofilm reactors. **Journal of Biotechnology** 80(1), 1-33.
- Nivens, D.E., Ohman, D.E., Williams, J., Franklin, M.J., 2001. Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. **Journal of Bacteriology** 183(3), 1047-1057.
- O'Toole, G., Kaplan, H.B., Kolter, R., 2000. Biofilm formation as microbial development. **Annual Review of Microbiology** 54(1), 49-79.
- Oh, H.S., Tan, C.H., Low, J.H., Rzechowicz, M., Siddiqui, M.F., Winters, H., Kjelleberg, S., Fane, A.G., Rice, S.A., 2017. Quorum quenching bacteria can be used to inhibit the biofouling of reverse osmosis membranes. **Water Research** 112, 29-37.
- Oh, H.S., Yeon, K.M., Yang, C.S., Kim, S.R., Lee, C.H., Park, S.Y., Han, J.Y., Lee, J.K., 2012. Control of membrane biofouling in mbr for wastewater treatment by quorum quenching bacteria encapsulated in microporous membrane. **Environmental Science & Technology** 46(9), 4877-4884.

- Ohlendorf, R., Vidavski, R.R., Eldar, A., Moffat, K., Möglich, A., 2012. From dusk till dawn: One-plasmid systems for light-regulated gene expression. **Journal of Molecular Biology** 416(4), 534-542.
- Palmer, J., Flint, S., Brooks, J., 2007. Bacterial cell attachment, the beginning of a biofilm. **Journal of Industrial Microbiology & Biotechnology** 34(9), 577-588.
- Papenfort, K., Bassler, B.L., 2016. Quorum sensing signal-response systems in Gram-negative bacteria. **Nature Reviews Microbiology** 14(9), 576-588.
- Paul, D., Kim, Y.S., Ponnusamy, K., Kweon, J.H., 2009. Application of quorum quenching to inhibit biofilm formation. **Environmental Engineering Science** 26(8), 1319-1324.
- Perni, S., Hackett, L., Goss, R.J., Simmons, M.J., Overton, T.W., 2013. Optimisation of engineered *Escherichia coli* biofilms for enzymatic biosynthesis of l-halotryptophans. **AMB Express** 3(1), 66.
- Peschke, T., Skoupi, M., Burgahn, T., Gallus, S., Ahmed, I., Rabe, K.S., Niemeyer, C.M., 2017. Self-immobilizing fusion enzymes for compartmentalized biocatalysis. **ACS Catalysis** 7(11), 7866-7872.
- Pesci, E.C., Pearson, J.P., Seed, P.C., Iglewski, B.H., 1997. Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. **Journal of Bacteriology** 179(10), 3127-3132.
- Petrov, G.I., Doronin, A., Whelan, H.T., Meglinski, I., Yakovlev, V.V., 2012. Human tissue color as viewed in high dynamic range optical spectral transmission measurements. **Biomedical Optics Express** 3(9), 2154-2161.
- Piatkevich, K.D., Subach, F.V., Verkhusha, V.V., 2013. Engineering of bacterial phytochromes for near-infrared imaging, sensing, and light-control in mammals. **Chemical Society Reviews** 42(8), 3441-3452.
- Pu, L., Yang, S., Xia, A., Jin, F., 2018. Optogenetics manipulation enables prevention of biofilm formation of engineered *Pseudomonas aeruginosa* on surfaces. **ACS Synthetic Biology** 7(1), 200-208.

- Purnick, P.E.M., Weiss, R., 2009. The second wave of synthetic biology: from modules to systems. **Nature Reviews Molecular Cell Biology** 10(6), 410-422.
- Qi, Y., Chuah, M.L., Dong, X., Xie, K., Luo, Z., Tang, K., Liang, Z.X., 2011. Binding of cyclic diguanylate in the non-catalytic EAL domain of FimX induces a long-range conformational change. **The Journal of Biological Chemistry** 286(4), 2910-2917.
- Rabaey, K., Rodríguez, J., Blackall, L.L., Keller, J., Gross, P., Batstone, D., Verstraete, W., Nealson, K.H., 2007. Microbial ecology meets electrochemistry: electricity-driven and driving communities. **The Isme Journal** 1(1), 9-18.
- Rabaey, K., Verstraete, W., 2005. Microbial fuel cells: Novel biotechnology for energy generation. **Trends in Biotechnology** 23(6), 291-298.
- Ramsey, D.M., Wozniak, D.J., 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. **Molecular Microbiology** 56(2), 309-322.
- Renicke, C., Schuster, D., Usherenko, S., Essen, L.-O., Taxis, C., 2013. A LOV2 domain-based optogenetic tool to control protein degradation and cellular function. **Chemistry & Biology** 20(4), 619-626.
- Rice, S.A., Koh, K.S., Queck, S.Y., Labbate, M., Lam, K.W., Kjelleberg, S., 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. **Journal of Bacteriology** 187(10), 3477-3485.
- Rittman, B.E., 1982. The effect of shear stress on biofilm loss rate. **Biotechnology and Bioengineering** 24(2), 501-506.
- Romling, U., 2005. Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. **Cellular and Molecular Life Sciences : CMLS** 62(11), 1234-1246.
- Romling, U., Gomelsky, M., Galperin, M.Y., 2005. C-di-GMP: the dawning of a novel bacterial signalling system. **Molecular Microbiology** 57(3), 629-639.
- Rosche, B., Li, X.Z., Hauer, B., Schmid, A., Buehler, K., 2009. Microbial biofilms: a concept for industrial catalysis? **Trends in Biotechnology** 27(11), 636-643.

- Ross, P., Mayer, R., Weinhouse, H., Amikam, D., Huggirat, Y., Benziman, M., de Vroom, E., Fidler, A., de Paus, P., Sliedregt, L.A., et al., 1990. The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives. **The Journal of Biological Chemistry** 265(31), 18933-18943.
- Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., de Vroom, E., van der Marel, G.A., van Boom, J.H., Benziman, M., 1987. Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. **Nature** 325(6101), 279-281.
- Rybtke, M.T., Borlee, B.R., Murakami, K., Irie, Y., Hentzer, M., Nielsen, T.E., Givskov, M., Parsek, M.R., Tolker-Nielsen, T., 2012. Fluorescence-based reporter for gauging cyclic di-GMP Levels in *Pseudomonas aeruginosa*. **Applied and Environmental Microbiology** 78(15), 5060-5069.
- Ryu, M.H., Fomicheva, A., Moskvina, O.V., Gomelsky, M., 2017. Optogenetic module for dichromatic control of c-di-GMP signaling. **Journal of Bacteriology** 199(18), 14-17.
- Ryu, M.H., Gomelsky, M., 2014. Near-infrared light responsive synthetic c-di-GMP module for optogenetic applications. **ACS Synthetic Biology** 3(11), 802-810.
- Sambrook, J., Russell, D.W., 2006. The inoue method for preparation and transformation of competent e. Coli: "ultra-competent" cells. **CSH protocols** 2006(1).
- Schirmer, T., Jenal, U., 2009. Structural and mechanistic determinants of c-di-GMP signalling. **Nature Reviews Microbiology** 7(10), 724-735.
- Schleheck, D., Barraud, N., Klebensberger, J., Webb, J.S., McDougald, D., Rice, S.A., Kjelleberg, S., 2009. *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. **PLoS One** 4(5), e5513.
- Shannon, M.A., Bohn, P.W., Elimelech, M., Georgiadis, J.G., Mariñas, B.J., Mayes, A.M., 2008. Science and technology for water purification in the coming decades. **Nature** 452(6599), 301-310.

- Sharma, G., Sharma, S., Sharma, P., Chandola, D., Dang, S., Gupta, S., Gabrani, R., 2016. *Escherichia coli* biofilm: development and therapeutic strategies. **Journal Of Applied Microbiology** 121(2), 309-319.
- Simm, R., Morr, M., Kader, A., Nimtz, M., Römling, U., 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. **Molecular Microbiology** 53(4), 1123-1134.
- Singh, R., Paul, D., Jain, R.K., 2006. Biofilms: implications in bioremediation. **Trends in Microbiology** 14(9), 389-397.
- Singh, V., 2014. Recent advancements in synthetic biology: Current status and challenges. **Gene** 535(1), 1-11.
- Sivakumar, K., Wang, V.B., Chen, X., Bazan, G.C., Kjelleberg, S., Loo, S.C.J., Cao, B., 2014. Membrane permeabilization underlies the enhancement of extracellular bioactivity in *Shewanella oneidensis* by a membrane-spanning conjugated oligoelectrolyte. **Applied Microbiology and Biotechnology** 98(21), 9021-9031.
- Spangler, C., Böhm, A., Jenal, U., Seifert, R., Kaefer, V., 2010. A liquid chromatography-coupled tandem mass spectrometry method for quantitation of cyclic di-guanosine monophosphate. **Journal of Microbiological Methods** 81(3), 226-231.
- Stewart, P.S., 1998. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. **Biotechnology and Bioengineering** 59(3), 261-272.
- Stewart, P.S., 2003. Diffusion in biofilms. **Journal of Bacteriology** 185(5), 1485-1491.
- Stewart, P.S., Franklin, M.J., 2008. Physiological heterogeneity in biofilms. **Nature Reviews Microbiology** 6(3), 199-210.
- Stewart, V., Yanofsky, C., 1986. Role of leader peptide synthesis in tryptophanase operon expression in *Escherichia coli* K-12. **Journal of Bacteriology** 167(1), 383-386.
- Stoodley, P., Hall-Stoodley, L., Lappin-Scott, H.M., 2001. Detachment, surface migration, and other dynamic behavior in bacterial biofilms revealed by digital time-lapse imaging. **Methods in Enzymology** 337(1), 306-319.

- Sudarsan, N., Lee, E.R., Weinberg, Z., Moy, R.H., Kim, J.N., Link, K.H., Breaker, R.R., 2008. Riboswitches in eubacteria sense the second messenger cyclic di-GMP. **Science** 321(5887), 411-413.
- Sutherland, I., 2001. Biofilm exopolysaccharides: a strong and sticky framework. **Microbiology** 147(1), 3-9.
- Sutherland, I.W., 2001. The biofilm matrix – an immobilized but dynamic microbial environment. **Trends in Microbiology** 9(5), 222-227.
- Tamayo, R., Pratt, J.T., Camilli, A., 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. **Annual Review of Microbiology** 61, 131-148.
- Tan, C.H., Koh, K.S., Xie, C., Zhang, J., Tan, X.H., Lee, G.P., Zhou, Y., Ng, W.J., Rice, S.A., Kjelleberg, S., 2015. Community quorum sensing signalling and quenching: microbial granular biofilm assembly. **NPJ Biofilms and Microbiomes** 1, 15006.
- Tang, L., Schramm, A., Neu, T.R., Revsbech, N.P., Meyer, R.L., 2013. Extracellular DNA in adhesion and biofilm formation of four environmental isolates: a quantitative study. **FEMS Microbiology Ecology** 86(3), 394-403.
- Tarutina, M., Ryjenkov, D.A., Gomelsky, M., 2006. An unorthodox bacteriophytochrome from *Rhodobacter sphaeroides* involved in turnover of the second messenger c-di-GMP. **Journal of Biological Chemistry** 281(46), 34751-34758.
- Teal, T.K., Lies, D.P., Wold, B.J., Newman, D.K., 2006. Spatiometabolic stratification of *Shewanella oneidensis* biofilms. **Applied and Environmental Microbiology** 72(11), 7324-7330.
- Tsoligkas, A.N., Winn, M., Bowen, J., Overton, T.W., Simmons, M.J., Goss, R.J., 2011. Engineering biofilms for biocatalysis. **ChemBioChem** 12(9), 1391-1395.
- Valentini, M., Filloux, A., 2016. Biofilms and cyclic di-GMP (c-di-GMP) signaling: Lessons from *Pseudomonas aeruginosa* and other bacteria. **The Journal of Biological Chemistry** 291(24), 12547-12555.
- Van Groenestijn, J., Geelhoed, J., Goorissen, H., Meesters, K., Stams, A., Claassen, P., 2009. Performance and population analysis of a non-sterile trickle bed reactor inoculated

with *Caldicellulosiruptor saccharolyticus*, a thermophilic hydrogen producer. **Biotechnology and Bioengineering** 102(5), 1361-1367.

Vigeant, M.A.-S., Ford, R.M., Wagner, M., Tamm, L.K., 2002. Reversible and irreversible adhesion of motile *Escherichia coli* cells analyzed by total internal reflection aqueous fluorescence microscopy. **Applied and Environmental Microbiology** 68(6), 2794-2801.

Wakimoto, N., Nishi, J., Sheikh, J., Nataro, J.P., Sarantuya, J., Iwashita, M., Manago, K., Tokuda, K., Yoshinaga, M., Kawano, Y., 2004. Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative *Escherichia coli*. **The American Journal of Tropical Medicine and Hygiene** 71(5), 687-690.

Wang, F., Gu, X., Zheng, C., Dong, F., Zhang, L., Cai, Y., You, Z., You, J., Du, S., Zhang, Z., 2017. Ehrlich reaction evoked multiple spectral resonances and gold nanoparticle hotspots for raman detection of plant hormone. **Analytical Chemistry** 89(17), 8836-8843.

Watanabe, T., Snell, E.E., 1972. Reversibility of the tryptophanase reaction: Synthesis of tryptophan from indole, pyruvate, and ammonia. **Proceedings of the National Academy of Sciences of the United States of America** 69(5), 1086-1090.

Waters, C.M., Bassler, B.L., 2005. Quorum sensing: Cell-to-cell communication in bacteria. **Annual Review of Cell and Developmental Biology** 21(1), 319-346.

Weerasekara, N.A., Choo, K.H., Lee, C.H., 2014. Hybridization of physical cleaning and quorum quenching to minimize membrane biofouling and energy consumption in a membrane bioreactor. **Water Research** 15(67), 1-10.

Weissleder, R., 2001. A clearer vision for in vivo imaging. **Nature Biotechnology** 19(4), 316-317.

West, S.E., Schweizer, H.P., Dall, C., Sample, A.K., Runyen-Janecky, L.J., 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. **Gene** 148(1), 81-86.

Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C., Mattick, J.S., 2002. Extracellular DNA required for bacterial biofilm formation. **Science** 295(5559), 1487.

Whitney, J.C., Colvin, K.M., Marmont, L.S., Robinson, H., Parsek, M.R., Howell, P.L., 2012. Structure of the cytoplasmic region of PelD, a degenerate diguanylate cyclase receptor that regulates exopolysaccharide production in *Pseudomonas aeruginosa*. **The Journal of Biological Chemistry** 287(28), 23582-23593.

Winn, M., Foulkes, J.M., Perni, S., Simmons, M.J.H., Overton, T.W., Goss, R.J.M., 2012. Biofilms and their engineered counterparts: A new generation of immobilised biocatalysts. **Catalysis Science & Technology** 2(8), 1544-1547.

Winzer, K., Hardie, K.R., Burgess, N., Doherty, N., Kirke, D., Holden, M.T.G., Linforth, R., Cornell, K.A., Taylor, A.J., Hill, P.J., Williams, P., 2002. LuxS: its role in central metabolism and the in vitro synthesis of 4-hydroxy-5-methyl-3(2H)-furanone. **Microbiology** 148(4), 909-922.

Wood, T.K., Hong, S.H., Ma, Q., 2011. Engineering biofilm formation and dispersal. **Trends in Biotechnology** 29(2), 87-94.

Wood, T.L., Guha, R., Tang, L., Geitner, M., Kumar, M., Wood, T.K., 2016. Living biofouling-resistant membranes as a model for the beneficial use of engineered biofilms. **Proceedings of the National Academy of Sciences of the United States of America** 113(20), e2802-e2811.

Wu, Y., Ding, Y., Cohen, Y., Cao, B., 2015. Elevated level of the second messenger c-di-GMP in *Comamonas testosteroni* enhances biofilm formation and biofilm-based biodegradation of 3-chloroaniline. **Applied Microbiology and Biotechnology** 99(4), 1967-1976.

Wu, Y., Mohanty, A., Chia, W.S., Cao, B., 2016. Influence of 3-chloroaniline on the biofilm lifestyle of *Comamonas testosteroni* and its implications on bioaugmentation. **Applied and Environmental Microbiology** 82(14), 4401-4409.

Wu, Y., Shukal, S., Mukherjee, M., Cao, B., 2015. Involvement in denitrification is beneficial to the biofilm lifestyle of *Comamonas testosteroni*: A mechanistic study and its environmental implications. **Environmental Science & Technology** 49(19), 11551-11559.

- Yang, L., Nilsson, M., Gjermansen, M., Givskov, M., Tolker-Nielsen, T., 2009. Pyoverdine and PQS mediated subpopulation interactions involved in *Pseudomonas aeruginosa* biofilm formation. **Molecular Microbiology** 74(6), 1380-1392.
- Yang, Y., Ding, Y., Hu, Y., Cao, B., Rice, S.A., Kjelleberg, S., Song, H., 2015. Enhancing bidirectional electron transfer of *Shewanella oneidensis* by a synthetic flavin pathway. **ACS Synthetic Biology** 4(7), 815-823.
- Yazawa, M., Sadaghiani, A.M., Hsueh, B., Dolmetsch, R.E., 2009. Induction of protein-protein interactions in live cells using light. **Nature Biotechnology** 27(10), 941-945.
- Yeon, K.M., Cheong, W.S., Oh, H.S., Lee, W.N., Hwang, B.K., Lee, C.H., Beyenal, H., Lewandowski, Z., 2009. Quorum Sensing: A new biofouling control paradigm in a membrane bioreactor for advanced wastewater treatment. **Environmental Science & Technology** 43(2), 380-385.
- Yeon, K.M., Lee, C.H., Kim, J., 2009. Magnetic enzyme carrier for effective biofouling control in the membrane bioreactor based on enzymatic quorum quenching. **Environmental Science & Technology** 43(19), 7403-7409.
- Yong, Y.C., Yu, Y.Y., Yang, Y., Liu, J., Wang, J.Y., Song, H., 2013. Enhancement of extracellular electron transfer and bioelectricity output by synthetic porin. **Biotechnology and Bioengineering** 112 (2), 408-416.
- Yu, H., Liang, H., Qu, F., He, J., Xu, G., Hu, H., Li, G., 2016. Biofouling control by biostimulation of quorum-quenching bacteria in a membrane bioreactor for wastewater treatment. **Biotechnology and Bioengineering** 113(12), 2624-2632.
- Zhao, E.M., Zhang, Y., Mehl, J., Park, H., Lalwani, M.A., Toettcher, J.E., Avalos, J.L., 2018. Optogenetic regulation of engineered cellular metabolism for microbial chemical production. **Nature** 555((7698), 683-687.
- Zhao, H., 2011. Highlights of biocatalysis and biomimetic catalysis. **ACS Catalysis** 1(9), 1119-1120.
- Zhao, S., Zou, L., Tang, C.Y., Mulcahy, D., 2012. Recent developments in forward osmosis: Opportunities and challenges. **Journal of Membrane Science** 396, 1-21.

Zobell, C.E., 1943. The effect of solid surfaces upon bacterial activity. **Journal of Bacteriology** 46(1), 39-56.