Engineering novel beneficial therapeutic microorganisms for *Vibrio cholerae* therapy using synthetic biology

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Thesis

Engineering novel beneficial therapeutic microorganisms for *Vibrio cholerae* therapy using synthetic biology

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"Veni, Vidi, Vici"

Julius Caesar in a letter to the Roman Senate

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Forever grateful,

The Author

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Abstract

Cholera disease is still a major global threat according to WHO. This notion is reinforced by the fact that the antibiotic resistance of *Vibrio cholerae*, the causative agent of the disease, is increasing in the epidemic strains. In year 2016 alone numerous occurrences of cholera outbreaks were reported, e.g. in Haiti and Sudan. The currently available methods of detection, treatment and prevention of cholera lack the required characteristics. Detection is slow or costly, treatment is mostly aimed at the symptoms and prevention in forms of vaccines is ineffective. To mitigate some of these problems, the goal of this research project is to engineer novel beneficial probiotic microbes to fight *V. cholerae* infections. To this end, a set of synthetic genetic circuits were created using the tools of synthetic biology. These circuits aim was to create a genetically modified *Escherichia coli* able to sense and kill *V. cholerae*. First, a whole cell *E. coli* based biosensor for *V. cholerae* detection has been designed, constructed and characterized. Second, this sensor circuit was redesigned to create sense and kill mechanism. Artilysin, a recently developed antimicrobial agent that can be directly expressed by cells was shown to be effective against *V. cholerae* cells. This work is first both to show a working *V. cholerae* biosensor in *E. coli* and an *E. coli* strain that is able to destroy *V. cholerae* upon its detection. Main advantage of this approach is that it should be possible not only to mitigate the cholera symptoms, but also to eliminate the cause of the disease, which was not possible before. This study is also a showcase of design principles for synthetic biology with examples of novel circuits with new, previously unknown applications and combinations. Finally, this integrated system forms a basis for a further development of viable detection and therapy option for *V. cholerae* infection.

List of abbreviations used in text

Art-085 – Artilysin 085

ATc – anhydrotetracycline

CTX – cholera toxin

CRISPR(i) – Clustered regularly interspaced short palindromic repeats
(interference)

dCas9 – dead Cas9

GFP – green fluorescent protein

gRNA – guide RNA

HCD – high cell density

IPTG – Isopropyl β -D-1-thiogalactopyranoside

LCD – low cell density

LuxOt – LuxO truncated

MIC – minimum inhibitory concentration

(t)pQrr – (truncated) Qrr promoter

RFP – red fluorescent protein

SN – supernatant

sRNA – small regulatory RNA

TCP – toxin coregulated pilus

Qrr – quorum regulatory sRNA

QS – quorum sensing

Chapter 1 Introduction

1.1 Cholera disease

Infection is invasion of human cells, tissues or organs by etiological agents such as bacteria, yeast or viruses and the host body reaction to such presence. Infectious disease is a disease which is the effect of an infection. Human body is able to fight most infections using its immune system, but unfortunately it is not always the case and sometimes medical intervention is required to prevent human health or life loss.

Cholera is potentially mortal, infectious disease known to humanity for centuries. The name of the disease comes from Greek word χολέρα (kholera), which is translated as “bile” [1]. The causative agent of the disease is the *Vibrio cholerae* bacterium.

1.1.1 Signs and symptoms

Cholera is a diarrheal disease caused by *Vibrio cholerae* bacterium. The most prevailing symptoms are vomiting and continuous, watery diarrhea (up to 1L per hour) causing severe dehydration. Fever is uncommon and is usually caused by a secondary infection. The symptoms start suddenly, after between 5 to 8 days of ingestion of bacteria (times as low as 12 h from ingestion were reported) [2]. The mortality of cholera can be as high as 80% if left untreated [1].

The characteristic watery stool caused by cholera is called “rice water” because of its resemblance to water left after boiling rice. Initially the excrements contain solid stool which is gradually replaced with fluid containing mainly water. If left untreated cholera cause severe dehydration caused by continuous secretion of water from the intestines. This is accompanied by electrolyte imbalance (mainly potassium and calcium ions). Severe cholera infection can cause death in hours from the first symptoms, especially in children. Due to the dehydration affected person usually develops secondary symptoms mainly connected with skin like wrinkling (due to lower skin turgor),

dryness of eyes and mouth. The patient can get dehydrated to a point where his/her skin becomes blue. The patient is often lethargic. All those symptoms are usually painless, save abdominal muscle cramps in some individuals. After hours of continuous defecation and vomiting, dehydration and electrolyte imbalance eventually lead to pulse irregularities and hypoglycemia. Death follows soon after.

1.1.2 Epidemiology

Cholera is transmitted through water contaminated with *V. cholerae* bacteria. In endemic areas *V. cholerae* is continuously present in water, which is its natural reservoir. During epidemics most of the bacteria in water comes from feces of cholera victims, both symptomatic and asymptomatic. This is why adequate sanitary measures are required to prevent cholera propagation – when sewage does not have an opportunity to taint clean water *V. cholerae* does not spread so easily.

John Snow was the first man to observe that the cholera infection comes from tainted water [3]. John Snow was a British physician, who was one of the pioneers of epidemiology. During cholera epidemic in London between 1848-1849 he proposed in a pamphlet “On the Mode of Communication of Cholera” that cholera is a waterborne disease, but it went by without much interest until 1854 when Snow persuaded London authorities that cholera is caused by tainted water [1, 3]. In the same year Italian scientist Filippo Pacini found, using a microscope, a comma like bacterium in a sample of stool of cholera victim. This bacterium, which was eventually named *Vibrio cholerae* was later isolated by Robert Koch, who published its detailed description [4].

Despite being known to the humankind for thousands of years, cholera still poses substantial threat. The 2013 WHO Report on Cholera reported nearly 130,000 cases of cholera which happened all around the world [5]. However, the same report pointed out that the number of cases is most probably under

reported as the estimated number of cholera cases worldwide is between 1.4 million to 4.3 million.

First mentions of cholera are found in Sanskrit texts from around 5th century BC [6]. It seems that cholera was first limited to the Indian continent, but then spread out eventually reaching Europe, Asia and the Americas. The historically first, endemic area of cholera is the Ganges delta in Bengal, India.

Cholera outbreaks can occur both in endemic and epidemic manner [6]. In the endemic form it's more dangerous to children than to adults and probably due to inherited immunity in many cases cholera has a mild course and up to 80% infections are asymptomatic. In epidemic setting cholera is as dangerous for children as for adults and is usually associated with very high mortality rate [6]. Also, epidemic strains of *V. cholerae* seem to be much more infectious than endemic ones, meaning the infectious dose is lower for epidemic strains [6].

Factors which contribute to cholera spreading have been identified as poor sanitation, malnutrition, poor healthcare and difficulties with administering the right treatment. Most developing countries are still struggling with the aforementioned and this is the reason why most of the outbreaks nowadays happen in less developed parts of the world.

The world has suffered from seven big cholera pandemics so far. The first one happened in years 1817-1824 and was limited to the Middle and Far East (first in India, then South-East Asia, eventually cholera reached Mediterranean Middle East). The second pandemic (1829-1837) again started in India, this time spread first to Russia and neighboring countries, by 1831 it reached western European cities of Danzig (now Gdansk), Berlin, Vienna and Hamburg. Ultimately, in 1832 it found its way into northern and then southern America. The third (1846 – 1855) and Fourth (1863 -79) pandemics encompassed the same lands as previous pandemics, reaching as far west as California. During the Fifth Pandemic (1881-1896), thanks to modernized sanitary measures, cholera's death toll in North America was much lower. The

death toll and spread of cholera in the Sixth (1899-1923) and Seventh (1961-1975) pandemics were very limited in Europe and North America attributed to development of modern sanitary systems. Nevertheless less developed parts of the world were still severely affected [1]. In the XXI century, despite major improvements in sanitary conditions worldwide there was one big epidemic encompassing Africa (Angola, Ethiopia, Somalia, and Sudan), Vietnam and Pakistan and the most recent and probably best known outbreak on Haiti which started soon after the 2010 Haiti earthquake.

Figure 1.1 Map of the world showing places with cholera cases with number of reported death cases in 2014. Adapted from [5] with permission.

Between the pandemics cholera was present in many parts of the world endemically. Besides parts of India, cholera was or is endemic in China, Bangladesh, parts of South-East Asia and South America. Without imposing strict sanitary regimes and improving the living conditions of those populations it will be very hard to eradicate cholera. The main problem with introducing better sanitary policies is the cost and time required for the construction of modern waterworks and sewage treatment systems in poor and developing countries.

1.1.3 *Vibrio cholerae* bacterium

Vibrio cholerae is a marine bacterium, Gram-negative and rod shaped. It is part of a larger family *Vibrionaceae*. It is a facultative anaerobe. It grows

best in media with high salt content, however it is able to grow also in low salt conditions as well [6]. In water *V. cholerae* enters a state in which it is uncultivable in laboratories, but it stays completely viable.

Figure 1.2 *V. cholerae* as seen under optical microscope. Photo taken by author.

V. cholerae are normally divided into over 200 serogroups, based on O antigen of the lipopolysaccharide, which is carried on the *V. cholerae* chromosome. The two infectious serogroups are O1 and O139, where based on biochemical and phenotypic characteristics the O1 serogroup can be further divided into classical and El Tor biovar [2]. Among many serotypes, the Ogava and Imaba ones are the most prevalent [6]. The classic O1 strain is believed to cause most of the epidemics in the past, replaced by O139 and O1 El Tor strains in XX century [6].

1.1.4 The *Vibrio cholerae* infection

The *V. cholerae* infection starts with ingestion of the bacteria. The infectious dose of O1 serotype of *V. cholerae* has been estimated to be between 10^5 – 10^8 [6, 7]. After the ingestion with tainted water, most of the bacteria die in the stomach due to its low pH. Those which survive, probably using the bacteria's pili, colonize the small intestines mucous membrane [2, 6, 8]. After

reaching the small intestines wall *V. cholera* adheres to the small intestines epithelium cells and starts expression of infection related proteins, among which the cholera toxin (CT) and toxin coregulated pili (TCP) are the most important.

The cholera toxin is an exotoxin secreted from the bacteria cell straight into the intestine lumen. This toxin consists of one A subunit and five B subunits. The A subunit makes the adenylate cyclase, enzyme responsible for producing cyclic AMP, to become constitutively on [6, 8]. This results in opening of chloride channels of intestinal epithelium cells, which leads to massive secretion of chloride ions and water [8]. The A subunit of the toxin enters the cells by assistance of the B subunits, which bind to the Gm₁ ganglioside on the surface of the epithelial cells [8].

During the toxin expression *V. cholerae* continues to divide to the point where the density of cells reaches a specified point. After reaching this point the gene expression profile changes. This can take between 2 to 14 days [6]. Cholera toxin is no longer expressed and instead proteins which facilitate cells evacuation from the intestine are being produced. This way cholera ensures its evacuation from the host in high numbers. The evacuation phase can take up to several days.

Genes directly responsible for *V. cholerae* infection (e.g. cholera toxin genes, *ctxAB*, and toxin coregulated pili genes, *tcpA-F*) are grouped in two separate gene clusters. One of them is the CTX ϕ filamentous bacteriophage (it is a prophage for *V. cholerae*), which can be incorporated in several positions in the *V. cholerae* genome (which is split into two chromosomes) [8]. This bacteriophage is transmittable between different *V. cholerae* cells with assistance of the TCP (toxin coregulated pilus). The CTX ϕ bacteriophage carries the CTX genes required for cholera toxin expression. Strains lacking the prophage are non-infectious, until they encounter cells with the phage and horizontal gene transfer will occur [9]. The CTX ϕ phage contents vary between

different strains of *V. cholerae*, giving the notion that the CTX ϕ phage can be itself divided into different strains [6]. The other cluster of cholera genes required for infection is so called vibrio pathogenicity island VPI-1 [6, 9]. This cluster includes the TCP genes and other required for infectious cycle signal propagation in the *V. cholerae* cells. Since the TCP is the receptor for the CTX ϕ phage, the *V. cholerae* strain has first to obtain VPI-1 and only then, after expressing TCP it is able to obtain the phage and start toxin production.

1.1.5 Diagnosis of cholera

Diagnosis of cholera is very important during the start of epidemics in a given area to alarm the respective authorities of the threat. World Health Organization in its manual “The treatment of diarrhea - A manual for physicians and other senior health workers” states that cholera should be suspected when the cause of death of patient 5 years old and older is severe dehydration due to watery diarrhea, even if the area is not known to be endemic with cholera. Cholera should also be suspected in a case where 2 years old or older patient develops acute diarrhea in an area known to have cholera [10].

Cholera can be diagnosed using standard laboratory identification methods using microscope and selective media and biochemical tests which are generally long and sometimes lack required selectivity. There are antibodies available for serotyping and serogrouping of *V. cholerae* [6]. Unfortunately, those methods require a laboratory to be available in the affected area.

Methods that can be used in places with limited laboratory access were also developed. Immunoassays for cholera toxin and *V. cholerae* LPS detection are available [11, 12]. Those immunoassays can be used for cholera infection detection directly from the patient’s stool. One of the commercially available immunoassays shows 97% sensitivity and 71-76% specificity towards *V. cholerae*, which is normally enough for reliable diagnosis [13]. However, the cost of such tests can be of concern.

1.1.6 Current Treatment

There are several treatment options available currently, including oral rehydration therapy, antibiotics and drugs which reduce secretion from the gastrointestinal tract (GI).

a) Oral Rehydration Therapy

The treatment of choice in cholera is the oral rehydration therapy (ORT). Early attempts of developing the right method were unsuccessful due to unfamiliarity with the electrolyte cotransport rules, specifically, that glucose has to be administered with sodium for effective absorption [6]. With the right administration of rehydration therapy, mortality of cholera can be as low as 0.2%. However, in many cases in regions plagued by cholera, there are not always conditions to perform this therapy optimally. The main problem is the availability of special salt formulations which after rehydration constitute the basis of ORT. Consequently, the mortality can be still around 10% [6]. Additionally, ORT does not kill *V. cholerae* cells which are free to infect next host.

The most challenging part of successful ORT is the estimation of the volume of fluids lost by the patient and which have to be replaced. It is often underestimated. On the average patients need 200 mL/kg of isotonic fluid in the first 24h hours from the onset of the symptoms [6]. The continued demand for fluid in later stages of the disease amounts to around 20mL/kg body weight/h of the isotonic fluid. In cases where the patient is unable to drink, intravenous solution can be used. However, this should be replaced with oral administration as soon as the patient is able to drink. This is because there are higher concentrations of ions in oral fluid than in the intravenous solutions [6]. The WHO supplies specially prepared sets of salts in form of powder for cholera treatment. In emergency such a solution can be prepared using standard household ingredients like sugar and salt.

b) Antibiotics

Antibiotics are used in cholera treatment as adjuvant therapy. The most widely used antibiotics used in *V. cholerae* infection are: tetracycline, doxycycline, ciprofloxacin, erythromycin and azithromycin [6]. As with many other bacteria nowadays, *V. cholerae* is showing more and more resistances against variety of antibiotics, especially against tetracycline and ciprofloxacin. It is worth noting that antibiotics cannot be used to fully cure cholera. Further, due to the growing reluctance of pharmaceutical companies towards developing new antibiotics, it is highly doubtful that a cholera curing antibiotic will be developed in the foreseeable future. Nevertheless, antibiotic therapy can reduce duration of the diarrhea and reduce fluid losses. It also reduces the time of bacteria presence in the infected persons stool [6].

c) GI secretion reduction drugs

Apart from antibiotics, another big group of medical compounds has its use in cholera treatment – drugs which reduce secretion from the gastrointestinal tract (GI). In this group of drugs one can find compounds such as inhibitors of enkephalinase enzymes, inhibitors of cystic fibrosis transmembrane regulator and agonists of peripheral opioid receptors (these are not exactly anti-secretory, but rather they reduce GI motility) [14]. When administered, drugs from those subgroups reduce the diarrhea, effectively reducing loss of fluids, which is lifesaving in many cases [14]. The main disadvantage of these drugs are their high cost and low availability in affected areas.

1.1.7 Prevention

Cholera outbreaks can be prevented by a variety of ways including improving sanitary and living conditions, vaccines and other, non- standard methods.

a) Improvement of sanitary and living conditions

Since cholera is a disease mainly connected to unsanitary living conditions, since XIX century there was a growing effort to alleviate this situation around the globe. The first effects of this could be seen during the fifth cholera pandemic, during which the number of cases and deaths was successfully reduced in more developed countries like Western Europe and Northern America. This situation persists nowadays, where most epidemics of cholera happen in the developing nations of Africa and Asia. For comparison, in 2014 there was a total of 17 cases of cholera in the whole of Europe, all imported, whereas Africa has seen as much as 105 thousand reported cases with mortality of almost 2% [5]. It is worth noting that these are only the reported and in most cases treated occurrences of the disease. Improvement of sanitary conditions is an on-going process in many developing countries, but it is estimated to take decades [6]. It is worth underlining, that even in regions where cholera is only endemic or absent, cholera epidemic can ignite very fast and with grievous outcomes, which was shown during the 2010 Haiti epidemic and again in more recent 2016 outbreak [15].

b) Vaccines

There is no fully effective vaccine against cholera infection. WHO is recommending two cholera oral vaccines – Dukoral and Shanchol [5]. As long as administration of the vaccines among affected communities can, arguably, help during epidemics, it has limited use as a preventive method in the long term. This is because the protection created by this vaccines declines rapidly after 6 months from the immunization. Generally, the expected protection level is 60-85% for 2-3 years after the immunization. WHO recommendation is to reimmunize populations in endemic areas to ensure protection persistence [6].

Dukoral, or WC/rBS is made by Crucell of Sweden. It is made of dead O1 strain *V. cholerae* cells with purified recombinant B subunits of the cholera toxin [6, 16]. The vaccine does not include subunit A of the toxin hence is safe for ingestion. It is recommended for people over 2 years old. It is administered in

two doses, normally 7-14 days apart, up to 6 weeks [5, 16]. The resistance usually develops after 1 week from the second dose. Its reported protection is 85% of cases in the first 6 months after administration, but it starts to fall sharply after this time to 58% in the second year. After three years, among immunized patients in Bangladesh the protection rate reported was about 35% [5, 16].

Shanchol or mCORVAX (names used in Vietnam and India respectively), the other dead cells vaccine recommended by WHO is made of both O1 and O139 strains cells, but without the B subunit of the toxin [5, 16]. Similarly, as with Dukoral, it is administered in two doses, with 2 weeks between them. It is recommended for all ages above 1-year-old. It has again the same shortcoming as Dukoral, two years after immunization the protection is only 67% [5].

Other vaccines, not recommended for use by WHO, but still licensed for use in certain parts of the world exist. Those include attenuated (unable to produce cholera toxin, thus safe for oral ingestion), genetically modified *V. cholerae* cells and comprise of CVD 103-HgR and Peru-15 among others. Those has proven to be safe and immunogenic, but as of yet were not proven to be effective in field [6, 16]. There also other vaccines, including both attenuated and dead cells vaccines, currently undergoing tests [16].

c) Methods using genetically modified organisms

With emergence of genetic engineering methods, it became possible to create microorganisms modified genetically so that they could fight cholera infection. Especially now, when synthetic biology gives us tools to systematically design such organisms one can expect novel cholera therapy methods based on genetically modified organisms to become available. One example of such effort would be the modified *E. coli* developed by Focareta et al. [17]. They have expressed glycosyltransferase genes, which bind cholera toxin, on the membrane of the *E. coli*. Such bound toxin is not able to affect epithelial cells of the intestines and hence they prevent water secretion. The main disadvantage of this solution is that it does not affect the *V. cholerae* bacterium itself which is

still free to divide, produce more infection related proteins and spread to other hosts. March and Duan on the other hand have developed a method using the quorum quenching mechanism [18]. Since *V. cholerae* produces the cholera toxin only when the density of cells in the environment is low they prepared a genetically modified *E. coli* that produced a chemical that “tricked” *V. cholerae* to switch to high density expression profile. This prevented *V. cholerae* from producing the toxin inside the intestines.

1.2 Objectives and Milestones

Given the fact that cholera is still present in many regions of the world and is claiming thousands of human lives every year it seems obvious that the current methods of treatment and prevention are not enough to stop the disease. Some of the many challenges standing against the modern world concerning infectious disease treatment and prevention in general are summarized below:

- a) Many of the major human pathogens which are causing infectious disease are gaining antibiotic resistance with worrisome pace. Even more distressing is the fact that the antibiotics themselves promote resistance in bacteria which means the more antibiotics are used the more resistant bacteria become. This fact coupled with the unwillingness of pharmaceutical companies to develop new antibiotics due to high development cost and low return rate could result in a return of pre-XX century times when even minor infection could result in death [19]
- b) Developing countries still struggle with delivery of treatment and prevention methods in many cases of infectious diseases. This situation often cannot be changed due to the costs of modern diagnostic, prevention and treatment options. Additionally, due to the high-tech nature of many of the drugs and vaccines production methods it is

infeasible for such production to be done by affected countries for cost cutting purposes.

- c) Due to a series of unfortunate publications and events, reluctance towards standard vaccines has greatly risen in recent years. Most prominently accusation of vaccines causing autism in young children attributed to this state of things. There is no scientific evidence for such correlation [20]. Because of this fact, many of the infectious diseases which were effectively under control due to vaccination efforts, such as mumps and measles, are again posing a threat.
- d) The growing global transport network (air, land and sea) is enabling infectious diseases to spread on an unprecedented scale [21]. As humans become more mobile in terms of their lifestyle, among many things they bring from the places they visit infectious diseases will undoubtedly be present. Transport growth is inevitable as it is an embedded quality of development. Therefore, humanity will have to come up with methods to prevent such disease spreading.

After taking the above facts into consideration, it becomes obvious that new methods of infectious disease control, prevention and treatment have to be developed. To address some of these challenges in terms of one the prevailing infectious disease - cholera, I propose to engineer therapeutics microbes that are capable of sensing and killing *V. cholerae*, by using synthetic biology approach. A genetically modified probiotic bacterium which would be able to sense and destroy *V. cholerae* cells *in vitro* will be developed. The detection of presence of the *V. cholerae* cells, will be achieved by detecting cholera specific compounds in the proximity of the genetically modified cells. After detecting pathogen presence, the modified cells will start expression of a killing molecule, to which the *V. cholerae* cells will be susceptible and should be readily killed by the said molecule. Moving forward, this system can be seen as a proof of concept for a probiotic bacterium to fight cholera infection within the human intestine.

To make *E. coli* able to detect *V. cholerae* presence, one of the natural quorum sensing systems present in *V. cholerae* was utilized. This system is normally used by *V. cholerae* to generate density dependent output. Specifically, *V. cholerae* protein expression profile is different when its concentration in a given medium is low than when it is high. That is possible, because (i) *V. cholerae* constitutively produces a series of specific compounds and secretes them into its environment [22-24] and (ii) each *V. cholerae* cell has special receptor proteins incorporated in their membranes, which can propagate appropriate signals into the cell upon the quorum sensing related compounds detection. The aim is to transfer this receptor and auxiliary proteins into the *E. coli*, intercept the signal along the way and redirect it into the expression of a killing module.

For *V. cholerae* killing some of the newly described killing molecules can be screened against it. These molecules should be possible to be expressed in *E. coli* so that they can be delivered at the target location.

The main advantages of the proposed approach are:

- a) The engineered probiotic is designed to be used as a preventative and curative measure since it will only produce killing molecules upon detection of *V. cholerae*
- b) The production of engineered probiotic could have substantial cost advantages owing to the relative ease of production in large scale bioreactors
- c) The engineered probiotic could be ingested in a similar manner as how existing probiotic bacteria are taken (e.g. yoghurts, drinks, capsules, dried forms, etc.), which can be made available in very affordable forms, readily distributed when necessary, and taken without the need for medical expertise/devices (e.g. presence of doctors, use of needles, etc.).

- d) By using non-antibiotic related method, the problem of antibiotic resistance can be overcome

These are important factors in favor of the adopted approach in developing countries where cholera is most prevalent.

1.3 Thesis Outline and Organization

This thesis presents the design and development of probiotic *E. coli* aimed at sensing and killing *V. cholerae*. Throughout the work, different parts of the final design were constructed and characterized. Eventually all the parts were integrated in one cell.

Chapter 1 introduces the reader to *V. cholerae*, the cholera disease and the currently used methods of cholera treatment and prevention. It explains why cholera is still an on-going, worldwide healthcare problem. It also specifies how the current problems with cholera could be alleviated by the designed *E. coli*.

Chapter 2 is the review of literature on the subject. First, the quorum sensing mechanism of *V. cholerae* is introduced. Next, currently known and useful in this design's case antimicrobial peptides and biosensors are reviewed. Further, the principles of synthetic biology are introduced. Finally, the chapter ends with description of CRISPR technology.

Chapter 3 describes the design and characterization of *E. coli* based *V. cholerae* biosensor. The division of the work into three different modules is explained and subsequently the work on each of them is described in detailed manner. Eventually, the characteristics of the final circuit are elucidated.

Chapter 4 discusses how the biosensor was turned into sense and kill system. First the testing of different antimicrobial peptides against *V. cholerae* has been described. Next, the integration of the biosensor with killing peptide is described.

Finally, chapter 5 discusses future works for the construct and chapter 6 gives detailed description of all laboratory techniques that were used during this work.

1.4 List of Publications

1. Holowko, M.B., et al., *Biosensing Vibrio cholerae with Genetically Engineered Escherichia coli*. ACS Synthetic Biology, 2016.

This article was also featured in Nature Chemical Biology as Research Highlight in November 2016 issue [25].

2. Jayaraman, P.*, Holowko, M.B.*, et al., *Repurposing a two-component system-based biosensor for the killing of Vibrio cholerae*. ACS Synthetic Biology, 2017.

* Authors contributed equally to this work

3. Holowko, M. B., et al., *Engineering beneficial microbes to sense Vibrio cholerae, human pathogen causing the cholera disease*. Presentation during International Conference on Biomolecular Engineering 2016 (ICBE 2016) in Singapore in January 2016
4. Holowko, M. B., et al., *Testing the specificity of an E. coli based V. cholerae biosensor*. Poster and lightening talk during IET/SynbiCITE Engineering Biology Conference in London in December 2016.
5. Holowko, M. B., *Biosensing Vibrio cholerae with Genetically Engineered Escherichia coli*. Invited to contribute chapter in Methods in Molecular Biology lab protocol series. Submitted to publisher.

Chapter 2 Literature review

2.1 Introduction

This chapter reviews the relevant literature related to the research. To begin with, introduction of quorum sensing concept, first in general bacteria and next in *V. cholerae* is addressed. Next, literature on antimicrobial peptides and proteins, different pathogen biosensors and concepts of synthetic biology are presented. Finally, CRISPR technology are reviewed to give a full understanding of the works presented in this thesis.

2.2 Quorum sensing

2.2.1 Quorum sensing in bacteria

Quorum sensing (QS) is a set of mechanisms by which bacterial cells communicate between each other. QS enables bacteria to avoid performing certain actions which are futile until the density of cells in a given medium reaches certain value. For example, biofilm forming bacteria, like *Pseudomonas* family, start biofilm formation only when enough bacteria are engaged in its formation. They do this because production of the biofilm does not make sense for single cell since not enough biofilm material could be produced as to provide full biofilm advantage [26]. To achieve such coordination in efforts, bacteria produce special compounds (simple organic compounds (ketones, aldehydes, alcohols and carboxylic acids) or short peptides [27]. These compounds are then detected by receptor enzymes which propagate the QS signal by either changing gene expression or by modifying other enzymes activity [28]. Two states in which bacteria can find themselves when referring to quorum sensing can be distinguished. Those states are called low-cell-density (LCD) and high-cell-density (HCD). In LCD individual, non-socially relevant processes are promoted. On the contrary, in HCD coordinated, socially justified actions are being carried out by the cells. Besides biofilm formation the QS systems are used for coordination of: protein secretion, bioluminescence, plasmid transfer, antibiotic production, virulence coordination and swarming motility with others most

probably awaiting their discovery [27, 28]. One genus of bacteria can use more than one distinct QS system.

Despite bacteria's enormous diversity, known QS systems can be divided into three major groups [27, 29, 30]:

- a) AHL based, found in Gram negative bacteria and used for intragenus communication
- b) Short peptide based, found in Gram positive bacteria and used for intragenus communication
- c) AI-2 based, found both in Gram negative and Gram-positive bacteria and used for intergenus and intragenus communication

Beside these three most prominent groups, more specific QS systems were found, with a notable case of *Vibrio* family of bacteria [30, 31]. Those can be used either for intergenus or very selective intragenus communication. The following paragraphs will give more details on the major QS systems groups with the detailed description of *Vibrio* QS following under the next headline.

Acyl homoserine lactones (AHLs) form the basis of the first big quorum sensing group. Found in Gram negative bacteria this system utilizes two main proteins for achieving density dependent state change. The first protein is the LuxI type enzyme responsible for AHL production [30]. The second protein is the LuxR type, DNA binding protein which uses AHL as a cofactor. Those names (LuxI type and LuxR type proteins) come from the original studies on this system in *Vibrio fischeri* in 1996 [32]. Since LuxI and LuxR are the QS part of the bioluminescence system in the *Vibrio fischeri*, other similar proteins in other bacteria incorporating AHL for QS are designated in the same fashion, e.g. in *Pseudomonas* family these proteins are named LasI (LuxI type protein) and LasR (LuxR type protein) [33]. The AHL mediated signal is species specific, that is, different bacteria species uses different variant of the AHL and are unable to process signals coming from AHLs secreted by other species. AHL after being produced by LuxI type enzyme is secreted to the environment. AHL is able to

penetrate bacterial membranes so it is directly detected by LuxR type protein which is confined in the cells cytoplasm. After binding the AHL LuxR type protein binds to its designated, highly conserved DNA sequence. After such binding promoter associated with this sequence is either activated or repressed [29].

Autoinducer 2 (AI-2) forms basis for the interspecies communication system between a variety of Gram positive and Gram-negative bacteria. AI-2 family of compounds is formed out of organic compounds, sometimes with metal ions included in the structure. The enzymes required for AI-2 synthesis (AI-2 synthases) form the LuxS type family of enzymes. They are found in variety of bacteria [34, 35]. There is much less variety in AI-2 types than in AHL. After active secretion AI-2 is detected by a specialized two-component receptor protein bound to the cells membrane which is able to propagate the signal inside of the cell incorporating a phosphorylation cascade [29, 36]. The exact role of the AI-2 mediated communication between different species bacteria is still unknown [34].

Gram positive bacteria communicate between each other using short peptides [27]. In contrast with the two other major groups of QS systems, the signaling peptides are usually encoded in the DNA and are directly expressed using the transcription/translation system. Since peptides cannot penetrate cells membranes, it is actively secreted and is usually coupled with the peptide modification such as cyclization [27, 30, 37]. After secretion, the peptide is detected by a two component sensor protein similar to the ones involved in AI-2 mediated communication. This sensor consists of membrane bound histidine kinase with one end exposed to the environment where the binding site is located. The other end, directed inside the cell is able to phosphorylate specific protein which then propagates the signal [36]. Since signal peptides are directly coded by the DNA there is a high variety of them. This coupled with the receptor protein ability to be activated only with specific peptide gives this system a high degree of specificity.

Bacteria can combine and modify the above-mentioned QS systems. It is common for bacteria to use both the AI-2 and AHL systems in parallel for interspecies and intra-species communication respectively. They are also able to intercept and disrupt signals coming from different cells, be it the same or other genus. This phenomenon is called quorum quenching [30, 38]. Since many bacteria use quorum sensing to coordinate medically and industrially important processes, like biofilm formation or antibiotic production, inhibiting quorum sensing looks like a valid approach to deal with such problems [38, 39].

Figure 2.1 Three types of quorum sensing in bacteria. A) AHL type of QS; blue shapes depict the AHL molecule that diffuses through the membrane to eventually bind to LuxR inside the cell B) short peptide type of QS; the green shapes depict the peptides that are transported outside the cell by specialized proteins and are being detected by sensor protein located on the membrane of the cell C) AI-2 (HAI-1 is a version of AI-2) type of QS, where HAI-1 and AI-2 diffuse outside the cell and are being detected by sensor protein located on the membrane of the cell. Adapted from [29] with permission.

2.2.2 Quorum sensing in *V. cholerae*

Vibrio cholerae shows two distinctive quorum sensing related states: low cell density (LCD) and high cell density (HCD) state. *V. cholerae* LCD state expresses the cholera toxin (CtxAB), proteins and other compounds connected with biofilm formation and other colonization associated proteins [27, 40]. This enables *V. cholerae* to efficiently colonize the host intestines which is the first step of the cholera disease. Toxin secretion and biofilm formation continues to a point when the population of bacteria is high enough to ensure that enough cells will be able to move to the hosts' environment with its stool. When this population level is reached, enough QS compounds are detected by *V. cholerae* receptors and the cell switches from LCD state to HCD. In HCD state *V. cholerae* stops cholera toxin production and other colonization linked proteins. Instead, production of numerous proteases is started which facilitates the cells detachment from the hosts intestines wall. This way the load of bacterial cells in the stool is greatly increased and *V. cholerae* increases the probability of getting into the environment [6, 23, 40, 41].

Vibrio cholerae employs a hybrid quorum sensing system for this density dependent expression control [24, 27, 42]. There is a number of known systems used for quorum sensing in *V. cholerae*, with most of them being based on two component sensor proteins [24, 27, 43, 44]. The QS systems of *Vibrio cholerae* have their counterparts in other species of *Vibrio* family [22]. Interestingly, regardless of which compound and respective receptor is generating the signal all of the systems converge to one protein called LuxO. Also, worth noting is that the system is heavily parallelized, meaning that: (i) the *V. cholerae* cells show density dependent state change even if only one out of the number of sensors is present (as long as LuxU and LuxO are left in the cell), (ii) there are four different sRNA (small RNA) expressed with help of LuxO, but they all work in the same way disrupting the same mRNA.

Figure 2.2 Proposed layout of the *V. cholerae* quorum sensing system. The solid shapes are the inducers. A) shows the state of the system when the autoinducers are absent. B) Shows the state of the system when the autoinducers are present. Adapted from [24] with permission.

The first of the well-established systems found in *V. cholerae* is the AI-2 dependent system known from other bacteria used for interspecies communication [31, 34, 45]. This system uses the LuxS protein for AI-2 production and LuxPQ proteins for signal reception. LuxP is a periplasmic protein able to bind AI-2 which cannot penetrate the inner cell membrane. LuxQ is a two-component system receptor which is a histidine kinase able to phosphorylate LuxU, phosphorelay protein shared with other QS systems in *V. cholerae* [24, 42]. LuxU then acts as a kinase and transfers its phosphate group to LuxO protein. In turn, the LuxO, coupled with sigma54 factor, can activate production of four small RNAs. These small RNAs, called Quorum Regulated RNA (Qrr), when expressed repress translation of another protein (HapR). HapR is able to activate and repress a number of dependent promoters in *V. cholerae* [46].

Second of the systems uses specific for *V. cholerae* genus autoinducer called cholera autoinducer 1 (CAI-1) [47]. It uses the CqsA protein for CAI-1 synthesis and CqsS membrane bound sensor protein for CAI-1 detection. CqsS, similarly to LuxPQ is a two-component histidine kinase which phosphorylates

LuxU for QS signal propagation. After the signal is relayed to LuxU, it follows the same path as in the AI-2 mediated system.

As shown before, the *V. cholerae* QS system comprises of more than the AI-2 and CAI-1 mediated QS pathways [43, 44, 48]. However, these systems are much less studied than the LuxPQ and CqsS systems. It is suggested that the influence of other hypothetical systems on the general QS of *V. cholerae* is small [24]. It is however worth noting that studies suggest that these other QS systems may converge with the AI-2 and CAI-1 ones in other parts of the signal propagation relay than LuxU, further parallelizing the system [24]. It is proposed that some of them may be working as deep in the relay as HapA (one of the proteins controlled by HapR) [48].

There is also a number of feedback loops which add an extra layer of control of the *V. cholerae* QS system [27] (Figure 2.3). The first feedback loop in the QS pathway involves LuxO. Specifically, LuxO, due to its gene arrangement can represses its own expression as it can bind to its own promoter. This is due to the fact that the Qrr1 gene and LuxO genes' promoters overlap and phosphorylate LuxO by binding to the activation site of pQrr1 hinders -35 region of its own promoter [49]. LuxO is also regulated by Qrrs themselves. This is done in similar fashion to disruption of mRNA of HapR by the Qrrs [50]. The other two discovered feedback loops are centered on the HapR protein. The first one involves HapR self-repression by binding to its own promoter [51]. The last of the known loops involve HapRs indirect activation of the Qrr promoters [52].

Quorum sensing system found in *V. cholerae* and other *Vibrio*, especially *Vibrio harveyi* are often found to be very similar and many parts shared by those two systems are homologous with small differences at minor points [27, 53, 54]. This implies that many findings in *Vibrio harveyi* are applicable in *V. cholerae* case.

Figure 2.3 Known feedback loops in the *V. cholerae* QS pathway. Adapted from [27] with permission.

2.2.3 Parts of Quorum sensing mechanism in *V. cholerae*

a) CAI-1 and AI-2

Structure of the CAI-1, one of the main compounds used by *V. cholerae* for QS was unknown for a long time. In 2007 Higgins et al. performed series of experiments which showed that CAI-1 is an organic compound whose systematic name is (S)-3-hydroxytridecan-4-one [31]. It consists of one long carbon chain and includes hydroxyl and ketone groups. Ng and Bassler in their review from 2009 suggest that different *Vibrios* produce a specific set of CAI-1 like molecules, which can be detected with different sensitivity by CqsS protein variants coming from different *Vibrios* [27]. Currently, CAI-1 variant used by *V. cholerae* is known to be synthesized and used by different bacteria from the *Vibrio* family, but it is sometimes suggested that in fact it is produced only by *V. cholerae* [47, 55]. Higgins et al. in the same work that elucidated the structure of CAI-1 also performed a series of experiments involving synthetic CAI-1 analogues to test CqsS specificity. Similar work was done by Bolitho et al. [56]. These works found that by modifying various parts of the CAI-1 molecule it is possible to obtain CAI-1 analogs which act as agonists and antagonists of CqsS. Additionally, analogues which activated CqsS more potently than the original

CAI-1 were found [55, 56]. Ng et al. reported the concentration of CAI-1 in a spent medium in which *V. cholerae* was grown equals to 222 nM [47].

Figure 2.4 Left: Structural model of CAI-1 variant used by *V. cholerae*. Adapted from [56] with permission Right: Structural model of AI-2 variant used by *V. cholerae*. Adapted from [57] with permission.

The systematic name of AI-2 used by *Vibrio cholerae* is (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate and besides containing tetrahydrofuran ring it also has a borate ion, which is rather unusual [31]. AI-2 is detected by LuxPQ tandem bound to the *V. cholerae* inner membrane. Different variants of AI-2 had been tested for probing the LuxP specificity [57, 58]. Lowery et al. tested LuxP from *Vibrio harveyi*, structurally similar to the one found in *V. cholerae*, with various AI-2 analogues. Enantiomeric, carbon chain and hydroxyl-group variants were among them. This study showed that LuxP from *V. harveyi* is capable of binding different versions of AI-2. However, if the structure of given AI-2 was different than the original used by *V. harveyi* the sensitivity of detection was substantially lower. This implies that LuxP is indeed specific towards the AI-2 used by *Vibrio* family [57].

b) CqsS

CqsS is a transmembrane histidine kinase. It is a part of two component system, which often involve histidine kinase enzymes similar to CqsS [59]. Apart from being a kinase CqsS also shows phosphatase activity. The target of CqsS phosphorylating and dephosphorylating activity is the LuxU protein, which than can transfer the phosphate group to another protein, LuxO.

Figure 2.5 The CqsS CAI-1 binding mechanism model. Adapted from [60] with permission.

In low cell density (LCD) state, when CqsS is not bound with CAI-1 coming from the environment CqsS autophosphorylates itself and acts as kinase towards LuxU [47, 55, 60]. This is done first by auto phosphorylation at histidine 194 (it's worth mentioning that CqsS acts in dimer configuration, so that histidine is cross phosphorylated by opposite dimers). The phosphate group is then transferred to aspartic acid residue 618. From there the phosphate group is transferred to LuxU histidine 58. In HCD (high cell density) CAI-1 accumulated in the environment binds to CqsS. This binding turns CqsS into a phosphatase which dephosphorylates LuxU. Wei et al. suggest in their article that actually the CqsS kinase and phosphatase activity is present simultaneously in the LCD, but the kinase activity is much more powerful. In HCD on the other hand, CAI-1 by binding to CqsS hinders phosphorylation at His58, which allows the phosphatase activity to dominate [60]. This change was deemed to be conformational [60].

The impact of CqsS mutations on its specificity was investigated. Ng et al. found that by changing the residues at position 104 and 107 CqsS preference towards different moieties at carbon 3 of CAI-1 can be changed. They have also shown that by changing residues 162 and 170 the preference towards different lengths and sizes of the head and tail parts of CAI-1 can be changed [47, 55].

c) LuxPQ

LuxPQ are two proteins closely cooperating in AI-2 detection. LuxP is a periplasmic protein which can bind both to AI-2 and to LuxQ. LuxQ is part of a two component system that is closely related to CqsS in function and structure. They also share the phosphorelay protein LuxU.

LuxP was demonstrated to be bound to the LuxQ kinase/phosphatase all the time rather than binding to it only after AI-2 is detected (bound to LuxP) [61]. Research done on *Vibrio harveyi* LuxPQ, organism highly related to *V. cholerae* shows that in LCD, when unbound by AI-2 LuxP is bound to LuxQ which makes it to act as a kinase [22, 62]. The kinase activity of LuxQ is similar to that of CqsS, meaning that LuxQ dimer first cross phosphorylate its histidines residues and then the phosphate group is transferred to aspartic residues. Finally, this phosphate group is moved to the histidine 58 on LuxU, exactly as it happened in the CqsS case [61, 62]. In the HCD situation, binding of AI-2 by LuxP changes the conformation of the whole LuxPQ complex so that the kinase activity of LuxQ is stopped and replaced by phosphatase activity [62]. This phosphatase activity is again directed towards LuxU.

d) LuxU

LuxU is a small, 113 amino acids long phosphorelay protein. Its role is to transfer the phosphate group between CqsS/LuxPQ and LuxO in both directions depending on the density state of the given cell. [63]. In LCD this phosphate group is first transferred between aspartate residues of CqsS/LuxPQ to histidine 58 of LuxU. The group is then transferred to aspartic acid 47 on the regulatory domain of LuxO [63]. In HCD this chain of reactions is reversed. LuxU was found to be partially homologous to other phosphorelay proteins involved in two component systems [59, 63].

e) LuxO

LuxO is a two domain protein which is the final acceptor of the phosphate group coming from CqsS/LuxPQ [27, 42]. The two domains consist

of: a regulatory domain which can be phosphorylated at aspartic acid 47 residue and a DNA binding domain which is of helix turn helix type (HTH) [64]. In the phosphorylated state the two domains are separated due to conformational hindrance [65]. This enables the HTH motif to bind to the Qrr promoters, which, together with the sigma⁵⁴ factor, promotes the Qrr synthesis. The binding region for LuxO in the Qrr4 promoter lies between -157 and -135 part of the promoter and consists of two binding sites [52]. In HCD state, when the CqsS/LuxPQ become phosphatases, LuxO is unphosphorylated and due to conformation change the two domains bind with each other [65].

Figure 2.6 The model of interactions of the LuxO domains in *V. harveyi*. D – regulatory domain, HTH – helix turn helix motif containing domain, LuxN – *V. harveyi* equivalent of CqsS. Please note that this system controls bioluminescence rather than virulence in *V. harveyi*, but otherwise both LuxO proteins are structurally similar. Adapted from [65] with permission.

LuxO can be permanently put in either the phosphorylated or unphosphorylated state by mutating a set of key amino acids or simply by removing the regulatory domain [65]. To obtain a LuxO mutant permanently mimicking unphosphorylated LuxO changing of 4th, 47th and 97th residue had been suggested. To obtain permanently active LuxO in terms of DNA binding, it is required to change the 47th or 94th residue or remove the whole regulatory domain altogether by shortening the LuxO gene [65].

f) Quorum regulatory sRNA

The quorum regulatory sRNA (Qrr) is one of the non-protein parts of the *V. cholerae* QS system. In total, there are four different Qrrs in *V. cholerae* genome. They have partially homologous sequence which, is highly conserved in region binding to the RBS (5' UTR) region of the HapR mRNA [66]. Each Qrr has a “built-in” terminator, that is their rho-independent terminators constitute part of the transcribed RNA. Their main role is repression of HapR translation by binding to its mRNA 5' UTR with assistance of Hfq chaperone protein [66, 67]. After such binding both Qrr and mRNA are degraded [68]. This ensures low HapR level in the cell and high Qrr turnover [50]. *V. cholerae* Qrrs are described as redundant, that is, if one, two or three are removed, the remaining one(s) will still enable the cell to maintain unaltered quorum regulated behavior (e.g. level of expression of cholera toxin which is directly coupled with QS is not changed even if three of the Qrrs are removed) [50]. Qrrs are divided into two distinctive parts, first part is used for HapR mRNA binding, while the second is used for Hfq binding [66].

Figure 2.7 Superposition of Qrr sequences over 5' UTR region of HapR mRNA. Adapted from [66] with permission.

Qrr expression is controlled by LuxO (the expression of Qrrs is possible only when the LuxO is phosphorylated) and σ^{54} factor [66]. Some research also suggests that Fis protein could be involved in Qrr promoters activation [43]. When the *V. cholerae* cell density in the environment is high enough and the cell switches to HCD state, LuxO is dephosphorylated and Qrr expression ceases. The expression level of different Qrrs in *V. cholerae* was reported to follow the following series: Qrr4>Qrr2≈Qrr3>Qrr1 [66].

V. cholerae Qrrs are often compared with a very similar system employed by *Vibrio harveyi*. It uses a very similar to *V. cholerae* QS system, but rather for controlling virulence it is used for bioluminescence control. However, in contrast to *V. cholerae* Qrrs redundancy, Qrrs used by *V. harveyi* (5 in total) are additive, which means that the bioluminescence control is hindered by removal of any number of the Qrrs [49]. This phenomenon is still not yet fully understood, but most of the research, done both in vitro and in silico suggest that this difference is due to different strength of the Qrr promoters in *V. cholerae* and *V. harveyi* and also due to differences in Qrr feedback loops [49, 52, 69, 70].

Figure 2.8 Qrr4 promoter sequence. Two LuxO binding sites along with -24 and -12 sites are shown. Adapted from [71] with permission.

g) HapR

HapR is also called a “master switch” protein, because of its ability to activate and repress a number of promoters. Those promoters control sets of genes where expression must be coordinated to ensure *V. cholerae* survival. HapR is part of a very diverse family of TetR repressors [53, 72]. HapR is homologous and interchangeable with *V. harveyi* LuxR [54]. This fact is often used during research on HapR, because LuxR is normally used for bioluminescence control in *V. harveyi* and one can transfer the bioluminescence operon into *V. cholerae* which is then controlled by HapR, providing an easy to measure change in phenotype [42]. The DNA binding domain of HapR contains a HTH motif [72].

In *V. cholerae* HapR is able to:

- a) activate HapA protein expression which is also called vibriolysin, a protease used by *V. cholerae* for detachment from human intestines [54]
- b) repress AphA protein expression which is a PadR family transcriptional regulator. AphA in tandem with AphB protein regulates expression of TcpPH genes, genes essential for *V. cholerae* virulence control [73]
- c) repress Vibrio expolysaccharide genes which are essential in biofilm formation [40]

The above information means that in LCD state, when HapR is not expressed (its translation is disrupted by Qrr binding), biofilm forming genes are expressed. Consequently, biofilm is formed. Additionally, AphA is expressed in the cell and it activates a cascade of gene expression which enables cholera toxin and other virulence linked genes to be expressed. In HCD state, when HapR expression is unhindered by Qrrs, AphA and biofilm genes cease to be transcribed. As a result, the cells become non-virulent. On the other hand, vibriolysin is being produced which enhances *V. cholerae* evacuation from the gut lumen.

Promoters activated and repressed by HapR were studied extensively [40, 51, 54, 73]. Apart from the genes listed above HapR can also bind and repress its own promoter [51]. It is worth mentioning that all these sequences are different which indicate that HapR can bind to a number of different sequences. However, these sequences have to be very specific as only one nucleotide change can prevent HapR from binding [73]. It is also suggested that different sequences have different binding affinity towards HapR [51].

2.3 Antimicrobial peptides

Nowadays the focus of antimicrobial research seems to shift from classic antibiotics to alternative means of fighting bacteria [74]. This is because the rising antibiotic resistance in bacteria is making classic antibiotics very ineffective in the fight with infections. Many new ideas are emerging to replace the antibiotics such as directed antibodies, phage-based therapies and

antimicrobial peptides. The review in this section will focus on the latter as they can be expressed directly by bacteria.

Bacteriocins are a diverse group of small oligopeptides produced by several species of both Gram positive and Gram-negative bacteria. Sometimes called very narrow spectrum antibiotics (there are, however, bacteriocins known to have quite broad spectrum), most of those peptides are killer molecules targeted against cells of the same or very genetically similar species to the producer species [75, 76]. Mechanisms of actions, structures and immunity strategies against bacteriocins are extremely diverse [75, 77]. One of the most prominent features is that their sequence is directly encoded in the bacterial genome or plasmids. Thus, they can be expressed using the normal transcription/translation machinery [75, 78]. Bacteriocins are usually divided into specific groups based on the degree of modifications they undergo. There are reports of using bacteriocins in probiotic bacteria for in situ treatment [79-81].

Microcins are a subgroup of bacteriocins produced by a family of Gram negative bacteria – Enterobacteriaceae [77, 78]. They show a relatively broad spectrum of action usually encompassing different species from Enterobacteriaceae family and related Gram negative organisms [77]. Like other bacteriocins, they are gene encoded small peptides with masses up to 10 kDa [77]. Microcin genes are often clustered with the corresponding immunity proteins [78, 82]. Microcins show a variety of killing mechanisms with pore and membrane potential disruption, replication and translation inhibition and membrane permeabilization among others [78, 83]. Microcin S is a class II microcin produced mainly by *E. coli*. The precursor oligopeptide is coded by the McsS gene, an immunity protein is coded by the McsI gene and the secretion system is coded by McsAB genes [82]. Zschüttig et al. isolated microcin S from the probiotic *E. coli* strain G3/10 [82]. Its exact killing mechanism is still to be elucidated, but with comparison to microcin L it can be predicted that it will be

by membrane disruption [84]. The McsI protein shows similarity in sequence to CAAX amino terminal protease family of enzymes [82]. The McsAB genes code for accessory protein are involved in Microcin S secretion and ATP-binding cassette transporter (ABC) [77, 82]. Microcin S was shown to be an effective killing molecule against *Pseudomonas aeruginosa* when secreted by probiotic *E. coli* [80].

Endolysins are a class of peptides produced by bacterial phages to lyse the host cells during their lytic cycle [85]. They work by degrading the peptidoglycan (PG) layer of bacterial wall. When applied from the outside of the bacterial cell, they are able to kill Gram positive bacteria. However, they are ineffective against Gram negative bacteria due to inability to go through their lipopolysaccharide (LPS) and outer membrane [86]. This shortcoming was alleviated by fusing an endolysin with an LPS puncturing peptide in a new class of promising antimicrobial peptides called the Artilyns [87, 88]. They are essentially fusion proteins made up of an endolysin and LPS puncturing peptide. Such a peptide retains the activity of both component peptides to permeabilize the cell membranes by disrupting both LPS and PG. An example of such an Artilyn is Art-085 made from SMAP-29 anti-LPS peptide from sheep blood and endolysin KZ-144 [89, 90]. SMAP-29 shows very high antimicrobial efficacy against a variety of bacteria [91]. The antimicrobial activity of KZ-144 is highly improved by such a fusion. Art-085 activity is the result of covalent fusion of the outer membrane-targeting peptide sheep myeloid 29-amino acid antimicrobial peptide (SMAP-29) to the N-terminal of the KZ144 endolysin. SMAP-29 can rapidly permeabilize Gram-negative bacteria's outer membrane and transport KZ144 through the outer membrane to degrade the peptidoglycan layer, thereby puncturing the cell. Compared to SMAP-29, Art-085 shows very similar antibacterial efficacy against a host of different bacteria species. However, it was not yet reported how effective it is against the *Vibrio* family. Additionally, SMAP-29 lyses human red blood cells whereas Art-085 does not [89].

Apart from peptides tested against general Gram-negative bacteria, there are also ones described as specifically being effective against *V. cholerae*. First of all a class of peptides called vibriocins could be distinguished, which are produced by some *V. cholerae* strains against other strains of the same genus [92]. Unfortunately, information about them is very scarce and does not enable their use in this research. Other peptides successfully used to kill *V. cholerae* include granulysin-based peptides and another type of fusion peptides called CM peptides based on cecropin and melittin [93, 94]. Granulysins, derived from human natural killer cells and show very high activity against a number of bacteria. With a small degree of modification they show high promise for antimicrobial action with negligible toxicity [94]. The CM peptides act by disrupting the bacterial membrane and similarly to Granulysins they show a high level of antibacterial efficacy with very low toxicity [93].

2.4 Pathogen biosensors

Diseases caused by bacteria are not always easy to diagnose, because humanity still lacks reliable, cheap and fast means to detect the microorganism responsible. The emergence of biosensor technology promises to change this dangerous situation. Biosensors use a biological element to sense a biological analyte and produce a measurable change. A more detailed description of biosensors was proposed by Adley et al. [95]:

“Biosensor is an analytical device, which converts a biological response into an electrical signal. It consists of two main components: a bioreceptor or biorecognition element, which recognizes the target analyte and a transducer, for converting the recognition event into a measurable electrical signal. A bioreceptor can be a tissue, microorganism, organelle, cell, enzyme, antibody, nucleic acid and biomimic etc. and the transduction may be optical, electrochemical, thermometric, piezoelectric, magnetic and micromechanical or combinations of one or more of the above techniques.”

Because so many different analytes can be sensed by the bioreceptors and at the same time systems output can be so diverse combinations of parts constituting a biosensor seem indefinite. To make this review more systematic the common classification system of biosensors based on the bioreceptor part will be used [95].

The first group of biosensors is based on enzymes as bioreceptors. Enzymes are natural candidates for bioreceptors as they are sensitive, usually very selective and with appropriate treatment stable for a long time. The main disadvantage is that a multi-step detection system is often required as the enzyme binding and processing the analyte rarely produces a directly detectable change. A wide variety of biosensors use an enzyme as an intermediate step of detection, but biosensors using enzymes directly as bioreceptors are used mainly for organic compound detection. One example of such a biosensor is a single enzyme nanoparticle based device to detect traces of urea in the environment developed by Zhang et al. [96].

Another group of biosensors uses antibodies for sensing. Since antibodies are highly specific and stable after immobilization, their use in biosensors is very common. One of their main drawbacks is the big effort required to produce antibodies for less common antigens [95]. Lim et al. constructed an antibody based biosensor for *Salmonella* detection [97]. In this biosensor quantum dot nanoparticles were coated with anti-*Salmonella* polyclonal antibodies. After binding the antigen the quantum dots were sorted using a microfluidic chip. Ravichandran et al. used a similar idea for the detection of *Vibrio cholerae* serogroups O1 and O139 [98]. Here, gold nanoparticles (AuNP) were functionalized with anti-*V. cholerae* monoclonal antibodies and then visualized using thin layer immunochromatography. Another example of an antibody based biosensor was developed by Waswa et al. [99]. This biosensor employed a gold surface which was able to detect

antibody - *E. coli* O157:H7 antigen binding by changing its refractive index due to surface plasmon resonance phenomenon.

The third big group of bioreceptors are the nucleic acid bioreceptors, usually DNA based [95]. DNA can be easily synthesized and it is easy to find a very specific sequence for the screened pathogen. In addition, DNA shows a wide range of biological and physical activities which can be used for signal transduction. The main disadvantage of nucleic acids is their high susceptibility to changes caused by various elements of tested samples. Chen et al. developed oligonucleotide functionalized AuNP to detect *E. coli* O157:H7 [100]. The DNA probe used in this biosensor was specific for the *eaeA* gene which had to be amplified using PCR before detection. A similar strategy was used by Chua et al. for *V. cholerae* detection [101]. Tichoniuk et al. showed that using self-assembled monolayer of thiolated DNA probes can be used for *Aeromonas hydrophila* detection because of the change of layers' electrochemical properties on analyte binding [102]. A very interesting detection strategy was chosen by Jie et al. [103]. Several different DNA probes against e.g. *Listeria monocytogenes* and *V. cholerae* were indirectly attached to part of the F_0F_1 -ATPase molecular motor. Upon binding of target analyte, the proton flux in the motor changed, which was detected by change in the fluorescence.

The final group of biosensors discussed in this review are whole cell based bioreceptors. Since cells react to multiple different stimuli and often produce a measurable change, they are also an interesting choice for the bioreceptor part of a biosensor [95]. However, there are severe drawbacks of using living cells as detectors – they need a controlled environment to sustain their life and additionally they multiply, which can generate various problems with the detection in time. Banerjee and Bhunia have developed a mammalian cell based (lymphocyte B) biosensor to detect pathogenic bacteria and their toxins [104]. The sensor was used for *Bacillus*, *Vibrio*, *Micrococcus* and *Serratia* bacteria and their toxins detection. Deng et al. employed a genetically modified

Pseudomonas syringae for the AHL detection [105]. Chang et al. have developed a genetically engineered *E. coli* which also was able to sense AHL produced by *Pseudomonas aeruginosa*, which could be potentially coupled with killing molecule production for *in situ* therapy [106].

Other methods, using non-standard techniques, have been developed. For example, Sunganak et al. have developed a method of *V. cholerae* O1 detection using atomic force microscope. Another example of such method would be development of synthetic vesicles for colorimetric detection of *E. coli* and *Staphylococcus aureus* [107].

To sum up, biosensors are very promising technology which can in the future enable easy and cheap detection of various pathogens. After finding ways to alleviate drawbacks showed by currently developed biosensors (low stability, high cost, and low availability) one can expect biosensor techniques to be universally adopted in various diagnostic kits, like in the modern pregnancy tests which already use biosensor technology.

2.5 Synthetic biology

a) Definition

Synthetic biology is a new discipline of engineering dealing with applying engineering principles to development of artificial biological systems [108]. The emergence of synthetic biology was possible due to development of DNA manipulation technologies, computational methods in biology, molecular biology techniques and related scientific and engineering disciplines. Despite being a young discipline, its creation was predicted soon after first molecular biology methods were developed. However, there is an ongoing debate on what kind of projects should be called synthetic biology. A common framework for synthetic biology project was laid out mostly during a Defense Advanced Research Projects Agency – sponsored study in 2003 [109]. It specified the workflow for synthetic biology projects:

- i. They start with design phase, where all the existing knowledge on explored and designed system is collected and then used for methodical design of a new, synthetic system
- ii. Genetic parts required for designed system are synthesized and prepared for assembly
- iii. Methodical assembly of the designed system from genetic parts
- iv. The project should conclude with systems testing and application phase

This framework, which is common for most engineering disciplines is followed by most synthetic biologists nowadays as it ensures rational design of new biological systems.

b) Achievements of synthetic biology

One of the most significant achievements of synthetic biology is the creation of open source repositories of standard biological parts and common standards of their assembly [110-112]. A genetic part is a DNA sequence with defined function. These parts can be made of naturally existing sequences in cells, like promoters, ribosome binding sites, genes, terminators and others. They can also be artificially enhanced or entirely man-made [108, 113]. Most of these parts are thoroughly characterized in their functions before being added to the repository. For example, for a genetic part which is a promoter, its strength compared to other promoters is examined and conditions for its activation are described (for example if it is constitutive or inducible) [113]. As for the assembly standards one of the first commonly adopted is the BioBricks standard created at Massachusetts Institute of Technology [110]. The main idea behind the BioBrick standard is that all the genetic parts compatible with it contains specially designed suffix and prefix sequences. Those suffixes and prefixes contain 2 restriction sites each (4 per part in total), which are used for parts ligation. Because of the specific selection of the restriction sites given parts can be assembled in series to create functional systems. There are modifications of the BioBrick standard where the prefix and suffix sequences are changed to

achieve a bit different functionalities, like for example in BglBrick standard which enables easier fusion protein creation [114]. The only requirement for vectors used with BioBrick and modified BioBrick standards is that they are free of restriction sites used in parts suffixes and prefixes [115].

c) Genetic circuits and their applications

Second very important achievement of Synthetic Biology are so called genetic circuits [116]. In analogy to electronic systems, genetic circuits are systems of genetic parts which combined produce some new function [116]. These functions are usually logical, meaning they output resemble executing of mathematical logical operation such as OR, AND or NOR [117, 118]. One of the first modern genetic circuits was the toggle switch created by Collins et al. [119]. It was described as a “synthetic, bistable gene-regulatory network”. It was made of two repressible promoters and two repressor genes which repress the promoter transcribing the opposite repressor. Since it is possible only for one of the repressors to be active in any given moment, by introducing a factor which would stop the currently produced repressor, the other one would start to be produced and eventually overcome the previous one. This way the working repressor is exchanged, and what’s more the new state is memorized by the system. Other circuits which include memorization of a given state by the cells were described by Eskin et al. [120] and Endy et al. [121] among others. The repressilator is another example of interesting genetic circuit [122]. In this circuit, genetic parts were combined to produce an oscillating level of three different repressors, where the oscillations repeat indefinitely in time. For more details please refer to Brophy and Voigt, who have recently published a very detailed review on genetic circuits [116].

Figure 2.9 The toggle switch design. Repressor 1 is induced by Inducer 1 and it inhibits promoter 1. Repressor 2 works analogically. Adapted from [119] with permission.

While synthetic biology currently concentrates around creating new capabilities in the lab, many model based computer software aiming in facilitating rational genetic part and circuit design were prepared. This include a ligand-binding proteins design software produced by Baker et al. [123], a metabolism change prediction software for designing a targeted overproduction by Maranas et al. [124] and ribosome binding site calculator by Salis [125] among others. Also, A.A. Nielsen et al. has recently come up with the CELLO software – an automated system for genetic logic gate design [126].

Since it was proven that the synthetic genetic parts and circuits can be combined with natural metabolic pathways existing in the cells, synthetic biologists started to look for practical uses of their discoveries and designs [127]. To achieve such combination a special interface circuit is required (please note that the parts described below are used not only for this interface) [128]. It may simply be a more or less complex promoter – repressor/activator pair which controls production of a protein engaged in one of the targeted organisms metabolic pathways [129]. At the same time, more advanced and complicated interfaces had been proposed and examined. Many of them are based on some form of RNA. The first example is the technology employing small regulatory RNA (sRNA), which coupled with chaperone protein Hfq binds to 5' UTR of target gene mRNA and disrupts its translation [130]. Other very interesting technology is CRISPR [131, 132]. In this system, a pair of Cas9 nuclease protein and a specially designed RNA element called the guide RNA (gRNA) work in tandem to

regulate target gene expression by activating, repressing it or changing its sequence. Riboregulators and riboswitches are yet another way to control gene expression using the RNA interactions [133, 134]. It is worth noting at this point that since many metabolic pathways in the natural cell can interfere with desired metabolite production there are attempts to create a “minimum” cell where all the metabolic pathways not required for maintaining life and desired production have been eliminated [135].

By combining genetic circuits with natural pathways many industrially and medically microorganisms have been produced. Synthetic biology is continuing work of genetic engineering, by providing producers of industrially important compounds. The difference is that synthetic biology instead of trying to randomly change the existing pathways adds new ones and explore the required outcomes *in silico* for more rational design. Using the synthetic biology approach strains producing hydrogen [136], various biofuels [137], glucaric acid [138], cadaverine [139] and many others were created. Many novel biosensors were also developed with the help of synthetic biology approach [140]. There are now successful attempts of combining biosensing and killing capabilities in one cell to allow “search and destroy” therapies [80, 106].

Figure 2.10 Example of genetically engineered *E. coli* for *P. aeruginosa* infection treatment. Constitutively expressed LasR protein after binding 3OC₁₂ HSL (AHL used by *P. aeruginosa* for QS) activated expression of pyocin (bacteriocin killing *Pseudomonas*) and lysis protein so that the pyocin gets released. Adapted from [106] with permission.

d) Future of synthetic biology

Synthetic biology has the great potential to become the next big thing in life sciences. Because of the circuit analogy it is easy to compare development of synthetic biology with the development of integrated electronic circuits. In this terms synthetic biology is still in its infancy. The most limiting factor in synthetic biology is the cost of DNA sequences synthesis. If the cost by base pair synthesis would be lower, more and more complicated systems could be produced faster and cheaper. But it seems that this time is finally coming [141]. When this problem will be overcome the possibilities for new synthetic systems are countless. A lot of effort is also recently put into automation of works in a synthetic biology laboratory. This is because many of the works like parts assembly and characterization of thereof are very laborious and give high hopes for good results with automation. It is easy to imagine one can make the cells to

perform any function that is physically possible. With this not only industry and medicine, but lives of all people can be changed.

2.6 CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) has been found in bacteria as a mean of cells self-defense system against virus and plasmid infection [142-145]. In short system works like this – each cell keeps an array of sequences that are corresponding to the foreign DNA (virus or plasmid) that may invade the cell in question (in case the cell is invaded with unknown virus or plasmid the cell has means to incorporate its “signature” into the array for future use). When the cell is invaded, special kinds of RNA (called crRNA and tracrRNA) are produced. After binding with the corresponding endonuclease, these RNAs guide the endonuclease to the target invading DNA to have it cut. As a result, the invasion is stopped.

The realization of how powerful tool this system can be came very fast [146]. By using crRNA and tracrRNA (which can be combined into single RNA called gRNA (guiding RNA)) and the endonuclease one can very efficiently cut DNA in a very specific spot determined by the gRNA sequence. Using this method, genomes can be easily manipulated in vitro or in vivo [147]. To do that the genome has to be exposed to the gRNA targeted to a specific site (or sites) and the nuclease. After the genomic DNA is cut, a replacement DNA can be introduced to replace the DNA that has been cut out using the CRISPR.

Cas9 is the most widely used nuclease for this purpose [148]. Also, in 2015 the Cpf1 nuclease has been found and its use is rising among CRISPR users [149]. The main difference between those two proteins is what protospacer adjacent motif (PAM) they recognize. PAM is a short, a few nucleotides long, sequence that has to precede the 20bp targeting sequence of gRNA for it to bind [150].

Figure 2.11 Comparison of CRISPR and CRISPRi techniques. Left branch shows CRISPR and right one shows CRISPRi. Adapted from [151] with permission.

CRISPR interference (CRISPRi) is a kind of derivative CRISPR technology where the Cas9 is mutated in a way that it is unable to cut the DNA, but is still able to bind to it [151-155]. This way the target DNA is not cut, but still is very efficiently bound by the gRNA-dCas9 complex. This can be used for expression repression. This is because once the gRNA guided dCas9 (dead Cas9, name underlining its inability to cut DNA) binds to promoter or the gene sequence it prevents the RNA polymerase to proceed with RNA synthesis [155]. This powerful tool enables selective silencing of genes in the genomes and plasmids. The big advantage of the system is that many genes can be silenced at the same time with high level of orthogonality by using different gRNA for each gene to be silenced [156].

The biggest concern when using CRISPR(i) is the possibility of off-target binding. This may lead to unwanted cuts in the genome in case of CRISPR or repression of genomic, potentially very important, genes in case of CRISPRi.

There are efforts to minimize this off-target binding, by rational design of the gRNA [157-160].

In summary, the CRISPR technology is possibly going to have a tremendous impact on the biological research [161, 162]. It is postulated that based on CRISPR a multitude of new gene editing/visualization technologies will emerge. This will include new gene therapies (by directly changing the genome in human cells with CRISPR), gene repression and activation technologies (genes can be activated by CRISPR if the nuclease is fused with an activator protein [152]) or genetic screening techniques in cells [163].

Chapter 3 Engineering *Escherichia coli* biosensor to sense *Vibrio cholerae*¹

3.1 Introduction

This chapter presents the development of whole cell biosensor for *V. cholerae* detection. It was decided that to construct such a biosensor, genetic modification of *E. coli* using part of the original quorum sensing mechanism existing in *V. cholerae* will be most practical. For reader's convenience, the *V. cholerae* QS system will be now quickly summarized as it will be much easier to understand the coming paragraphs describing design of the circuit.

The CqsS/LuxPQ sensor protein, when not bound by CAI-1/AI-2 respectively (this is the case when this compound, produced by *V. cholerae*, is in low numbers in the environment) phosphorylates indirectly (through LuxU protein) LuxO protein. Phosphorylated LuxO, in tandem with σ^{54} factor, activates Qrr (quorum regulated sRNA) expression. The Qrrs (there are 4 in total) are able to repress HapR translation. In result, HapR is not present in the cell and pAphA, a constitutive promoter repressible by HapR protein, is constantly active. When CAI-1 is in high numbers in the environment (*V. cholerae* density in the medium is high) the whole chain is reversed and the pAphA is repressed by HapR. So, when CAI-1/AI-2 concentration in the environment is high target gene under pAphA is not expressed and, on the other hand, the target gene is highly expressed in low CAI-1/AI-2 concentration.

From two candidate sensor proteins, CqsS and LuxPQ, the former was chosen for the sensor construct. That is because LuxPQ is sensitive to AI-2 molecule, which is not very specific to *V. cholerae* [31]. On the other hand, reviewed articles suggest that CAI-1 is a family of closely related compounds used only by *Vibrio* family and the variant of CAI-1 used by *V. cholerae* is very specific to it and its version of CqsS receptor protein. As the CqsS is not a DNA

¹ Part of this chapter was published in ACS Synthetic Biology [Holowko, M.B., et al., *Biosensing Vibrio cholerae with Genetically Engineered Escherichia coli*. ACS Synthetic Biology, 2016]. Reprinted with permission. Copyright 2016

acting protein (it doesn't alter gene expression directly, moving of LuxU and LuxO proteins for CqsS signal transduction was also required. LuxO with sigma54 factor is responsible for Qrr promoters' (pQrr) activation, so it is planned to move one of those promoters into the designed construct and put a target gene under its control, so it will be activated by LuxO. This means that in absence of CAI-1 (LCD state) in the medium the system will produce the protein under pQrr promoter (it will be called pQrr target protein from now on). Then, with increasing CAI-1 concentration in the medium (transition from LCD to HCD state) pQrr target protein level in the cell will gradually fall (Figure 3.1).

Figure 3.1 Illustration of final engineered *E. coli* based *V. cholerae* biosensor working principle when the circuit is activated by CAI-1 produced by *V. cholerae*. CAI-1 by binding to CqsS results in its dephosphorylation followed by LuxU and LuxO dephosphorylation. Dephosphorylated LuxO is unable to activate expression of the repressor, which leads to unhindered expression of GFP. Elements of the sensing module are shown in blue, inverting module is shown in red and actuating module is shown in green. The full genetic circuit diagram can be seen in Fig. 3.27.

The obvious consequence of this is that the pQrr target protein is being produced when no *V. cholerae*/CAI-1 is present in the medium and its production is stopped when the *V. cholerae*/CAI-1 concentration in the medium goes up. This means that it is not possible to put the killing molecule gene under direct control of pQrr promoter. To overcome this problem a genetic inverter part will be required, which in this case would mean expressing a repressor as the pQrr target protein and then the actual killing molecule under the promoter repressed by the aforementioned repressor. For this genetic inverter a few

different parts can be considered. Possibility one is using the natural *V. cholerae* inverter based on HapR and the accompanying Qrr molecules. Another option would be designing and characterizing a new inverter based on CRISPRi technology. The final option would be using one of the widely known TetR like repressors for this purpose [164].

Figure 3.1 shows in a graphical form the working principle of the *E. coli* biosensor. To rationalize the design of the construct in the *E. coli* it was decided to break up construction and characterization of *V. cholerae* sensing system into three modules (Figure 3.2):

- a) The CqsS:LuxU:LuxO:pQrrX based sensing module where a fluorescent protein (RFP or GFP) will be expressed under pQrrX (Qrr promoter, where X is the Qrr number) constitutively. It will be possible to lower this expression with addition of CAI-1 compound in different concentrations into the growth medium of the bacteria. This will allow us to thoroughly characterize this part of the sensor using standard fluorescence measurement techniques. An *in silico* model of this module was developed to aid the design process by eliminating part of the otherwise required combinatorial construction work.
- b) For the inverting module, it was needed to consider, design and characterization of a set of different inverters to meet the right requirements for such an inverter, namely being able to show distinguishable ON and OFF state and also showing as low as possible residual expression
- c) The actuating module which in this case constitutes already well characterized Green fluorescent protein (GFP) and the promoter used of its expression

It is worth noting that the modules have two interfaces. The first interface between the Sensing and Inverting module is the pQrrX promoter, because on one hand it's being activated by the LuxO, but on the other hand it

controls expression of the Inverter module genes. The second interface is between Inverting and Actuating module. This interface is again a promoter and this time the promoter is controlled by the repressor since it used for the actuating molecule expression.

After successful testing of all the modules of the system, that is the sensor, inverter and actuator were combined into one whole system and moved into one *E. coli*. As a proof of concept, in this thesis, all the construction and testing stage constructs were done in TOP10 and MG1655 strains.

Figure 3.2 Simplified diagram from Figure 3.1 showing how the main design is divided into its constituent modules. Please note that the modules interface at genes.

3.2 Characterization of the actuating module

To study the sensor's (CqsS, LuxU and LuxO) performance in *E. coli*, it was first required to find a suitable reporter protein coupled with LuxO dependent promoter. Being a well-established and easy to use reporter protein, green fluorescence protein GFPmut3b was chosen [165]. LuxO, a transcription factor protein, activates four different promoters in *V. cholerae*, one for each Qrr molecule. This activation is done by phosphorylated LuxO binding to $\sigma 54$ factor of the RNA polymerase. Interestingly, the phosphorylated (active) state of LuxO can be mimicked by removing its regulatory domain [166]. In this study, this

truncated LuxO (LuxOt) was used to mimic the activated state of LuxO. Among all the Qrr promoters, pQrr4 (sequence as shown in Figure 3.4) which is responsible for expressing sRNA Qrr4 is the best studied one and it shows quite high expression relative to other Qrr promoters, so it was decided to test its feasibility for use in this study [71].

Figure 3.3 Graphical depiction of the pQrr4 testing circuit. LuxO(t) expression is activated with addition of arabinose and as a results the GFP expression is started from the (t)pQrr4. Reproduced from [167] with permission.

Figure 3.4 Sequence of the Qrr4 promoter. LuxO binding sites are highlighted in red. Sigma factor binding sites (-24 and -12) are underlined. Part of the sequence that constitutes truncated Qrr4 promoter is bolded and showed in blue. Reproduced from [167] with permission.

To elucidate the performance of pQrr4 in *E. coli*, the pQrr4 was characterized by expressing LuxO (inactivated state) or LuxOt (activated state) under pBAD arabinose promoter from plasmid which also harboured GFP under control of pQrr4 (Figure 3.3). As the testing gene circuit does not include CqsS and LuxU, LuxO would stay unphosphorylated in the cell. pQrr4 is expected to only turn on when phosphorylated LuxO is present and remain off otherwise. However, the results show that the pQrr4 is not selective towards phosphorylated LuxO as the promoter is activated when LuxO is expressed (Figure 3.5). Further, when LuxOt is expressed in the same setup the GFP expression is 2/3 times higher than in the case of LuxO (Figure 3.5, second and fourth bar from the left). This implies that although pQrr4 is activated stronger

by phosphorylated LuxO, it retains some of the activity with unphosphorylated LuxO present alone. To overcome this problem, it was decided to shorten the pQrr4 so that it only consists of the required minimum – binding sites for σ_{54} and phosphorylated LuxO. It was previously shown that for LuxO type activators the promoter is still activated in presence of the activator after removing the activator binding sites [168]. The truncated pQrr4 (tpQrr4 - Figure 3.4) was tested with both LuxO (inactivated state) and LuxOt (activated state) using the aforementioned circuit (Figure 3.3). Results show that although the tpQrr4 is much weaker compared to the full original pQrr4, it is much more selective towards LuxOt which mimics phosphorylated LuxO. Consequently, tpQrr4 was chosen as the promoter for further study.

Figure 3.5 Qrr4 promoter characterization. A) Comparison of GFP/OD600 readings after 4 hours of microplate cultures for all constructs. Truncated pQrr4 is much weaker, but is not activated by dephosphorylated LuxO. B) and C) Transition curves showing change in GFP/OD600 level in both full (B) and truncated (C) pQrr4 in different arabinose

concentrations. The levels shown are after 4 hours of culture. Data is fitted with Hill type of curve, r square for all fits > 0.95. Reproduced from [167] with permission.

3.3 Design and characterization of inverting module

3.3.1 Introduction

As was mentioned in subchapter 3.1, the signal coming from the sensor module must be inverted. That means that when the CAI-1 is detected the circuit should start production of effector proteins rather than stop which would be the case if the final promoter of the system would be pQrr4. Since tpQrr4 has been shown to be a fairly weak promoter, it was needed to amplify the GFP expression as well. Consequently, there was a need for an inverter that could invert and amplify the signal from the CqsS-LuxU-LuxO sensing cascade. To this end, the natural Qrr based inverter from *V. cholerae* is studied, then TetR family based repressors are considered and finally a new genetic inverter based on CRISPRi technology is designed and characterized.

3.3.2 Qrr based inverter from *V. cholerae*

The natural inverter in *V. cholerae* cell comprises a set of four Qrr molecules, HapR repressor protein and corresponding promoters. The Qrrs when expressed stop HapR expression by binding to its mRNA and thus rendering it unusable. HapR protein, when it is expressed represses AphA protein promoter and activates promoter for HapA protein. It is also able to induce expression from the promoter for the Lux operon (pLux) from *V. harveyi* [31]. So, if it would be decided to move the original system into the designed system it would require moving LuxO, one of the Qrrs and HapR with corresponding promoters into the modified cell. This cascade would work as follows – LuxO would control expression of the chosen Qrr to turn the expression of HapR ON and OFF. Since the repression capability towards AphA promoter (pAphA) is well described in the literature, it was decided to first characterize its performance rather than the activated promoter. So first it was required to characterize the pAphA and its behavior in *E. coli*, then examine how

good is the HapR expression of it and finally which of the four Qrrs show best capability to stop HapR expression.

The pAphA promoter was characterized to determine promoter strength and dynamic behaviour. To study the characteristics of the AphA promoter in *E. coli*, a plasmid was constructed in which red fluorescence protein (RFP) transcription was driven by pAphA. To prevent any influence from a leaky pBAD promoter upstream of RFP, a terminator between pBAD and pAphA was introduced. Kovacikova et al. described the AphA promoter in great detail, but the exact mechanism of its repression by HapR has not been investigated [73]. Additionally, although their work showed that the promoter functions in *E. coli*, the dynamic behaviour of pAphA was not demonstrated, nor was the pAphA promoter compared with other commonly used promoters in terms of promoter strength. To address these uncertainties, a normalized test was performed demonstrating pAphA strength and dynamic behaviour as compared to other widely used *E. coli* promoters.

Figure 3.6 Comparison of normalized RFP fluorescence divided by OD₆₀₀ for AphA promoter and other commonly used promoters. Additionally, the expression level from the pAphA with supposedly original HapR protein RBS only is showed.

Figure 3.6 depicts the levels of normalized RFP fluorescence (RFP fluorescence divided by OD₆₀₀ of the culture) produced by different promoters in TOP10 *E. coli* with plasmid carrying pAphA directly upstream of RFP gene. This result was compared with RFP fluorescence per OD₆₀₀ of cultures harbouring

similar plasmids but with an *rrsBP1* (BBa_K112118), BBa_J23108 or BBa_J23105 promoters instead of pAphA. These promoters are widely used in *E. coli* [111]. A plasmid carrying the pAphA have also been constructed, but with the ribosome binding site (RBS) upstream of RFP in pBbE8k (the backbone plasmid) removed to ensure that in the cloned sequence encompassing the pAphA promoter, there is no RBS sequence which would influence RFP expression levels. These results shows that pAphA is a medium strength, constitutive promoter. pAphA is 15% stronger than promoter *rrsBP*, which is commonly used as a reference promoter [169], and is 50% and 270% stronger than BBa_J23108 and BBa_J23105 respectively. RFP expression from the plasmid where the RBS upstream of RFP in pBbE8k was removed has been shown to be minimal and negligible.

To fully characterize the influence of HapR on pAphA in *E. coli*, the HapR gene was added to the plasmid used for pAphA characterization (Figure 3.7). This HapR was expressed under an arabinose-induced promoter (pBAD). As a result, the amount of HapR produced could be controlled and its efficacy in repressing pAphA established by measuring the reduction in RFP fluorescence per OD₆₀₀ of the cultures induced with different arabinose concentrations. The readings were compared after the cultures reached stationery phase. Two different variants of HapR were tested, one from *V. cholerae* O1 El Tor which is a virulent strain of *V. cholerae* and the second from an O37 serotype which does not cause cholera disease in humans [170]. The only difference between these genes is a point mutation in 116th base - there is a guanine in that position in El Tor, whereas there is an adenine in O37. This mutation changes glycine (El Tor) into aspartic acid (O37). Thus, instead of a very small residue (hydrogen) there is a big, acidic residue which could potentially disrupt the TetR binding motif of HapR where this mutation occurs. Both variants of the HapR protein were tested to see if this mutation affects HapR repression capabilities. In the following paragraph, the plasmids carrying El Tor O1 HapR and O37 HapR are referred to as pBAD-HapRO1-pAphA-RFP and pBAD-HapRO37-pAphA-RFP respectively.

Figure 3.7 Graphical depiction of the HapR/pAphA testing circuit. HapR expression is activated with addition of arabinose and as a results the RFP expression is repressed by HapR binding to the pAphA.

Figure 3.8 shows the comparison of results of cultures of TOP10 *E. coli* carrying either pBAD-HapRO1-pAphA-RFP or pBAD-HapRO37-pAphA-RFP constructs with different arabinose concentrations in the medium. The results show that 0.05% arabinose in the growth medium is sufficient to achieve maximum HapR repression for both O1 and O37 HapR versions (Figure 3.8), although this maximum repression differs between O1 and O37 HapR. It is clearly visible that the point mutation described above significantly lowers O37 HapR repression of the pAphA promoter. Fully-induced cultures with O1 HapR show more than 70% reduction of the normalized fluorescence of non-induced cultures, while O37 shows less than 20% of reduction of the normalized fluorescence of the non-induced cultures. This means that the O37 variant of HapR could be eliminated from further study as unsuitable for the inverter. However, another important feature of O1 HapR expression in *E. coli* has emerged. Namely, expression of O1 HapR lowered significantly the growth rate and final OD₆₀₀ of the cultures (Figure 3.9).

Figure 3.8 Comparison of normalised RFP/OD₆₀₀ for cultures expressing O1 HapR or O37 HapR. Without arabinose cultures with O1 HapR have somewhat higher expression, however, after addition of arabinose the inability of O37 HapR to repress the pAphA efficiently becomes evident.

Figure 3.9 Results of detailed O1 HapR characterisation. A) Plot showing change in RFP/OD₆₀₀ and OD₆₀₀ of cultures with different arabinose concentrations B) Change in RFP

fluorescence/ OD_{600} of culture not expressing (0% arabinose) and expressing HapR in high amounts (0.05% arabinose). C) Plot showing the same as in B), but in terms of OD_{600} change

To investigate the efficacy of HapR translation disruption by Qrr, a plasmid was constructed with the addition of one of the four Qrrs and the constitutive promoter (*rrsBP1*) to pBbE8k with HapRO1-pAphA-RFP (which carries O1 HapR with original HapR RBS in front of HapR) (Figure 3.10A). This allows HapR to be transcribed constitutively under *rrsBP1* (which has been proven to be effective in terms of pAphA repression without significant OD_{600} decrease) and the chosen Qrr induced by addition of arabinose. The Qrr translation disruption efficacy was tested using the original HapR RBS because Qrrs bind specifically to the RBS of HapR, so retaining this sequence is required for effective binding of Qrrs to the HapR mRNA. The sequence and estimated structures of the four Qrrs can be seen in Figure 3.11. In total, four different plasmids were prepared, each carrying one of the Qrrs. In these constructs, constitutively expressed HapR represses the AphA promoter. Hence, RFP production was maintained at low levels. After addition of arabinose into the growth medium, Qrr is expressed, thereby disrupting HapR translation which reduces the amount of HapR in the cells (Fig. 3.10A). Consequently, RFP levels in cells will increase.

Figure 3.10B,C shows the relative Qrr efficacy in disrupting HapR mRNA translation measured as RFP fluorescence per OD_{600} level of cultures with different arabinose concentrations added to the medium, normalized against the RFP fluorescence of cultures without arabinose added (i.e. fluorescence of culture of given arabinose concentration is divided by fluorescence of culture with no arabinose added). This data shows that Qrr4 has a much higher efficacy than other Qrrs. Qrr4 disruption of HapR mRNA occurs at lower arabinose concentrations. Furthermore, Qrr4 after full activation achieves two times higher RFP fluorescence per OD_{600} levels than cultures expressing any other Qrr.

Figure 3.10D shows changes in the RFP fluorescence per OD_{600} over time of microplate cultures of pBAD-Qrr4-*rrsBp1*-HapRO1-pAphA-RFP uninduced,

partially induced (0.001% arabinose) and fully induced (0.05% arabinose) [data for other Qrrs were similar, data not shown]. Two observations from these results can be made. Firstly, Qrr4 expressed under fully activated pBAD (which is a strong promoter) after achieving stationary phase results in an increase in RFP fluorescence per OD₆₀₀, which implies that there is a decrease in the HapR concentration in cells. Secondly, there is a delay (approximately 2h) after addition of arabinose and an observable increase in fluorescence in the Qrr-expressing culture, compared to the uninduced culture. This could be due to HapR stability, as Qrr4 would not affect HapR proteins that were already present in the cell, as Qrr4 only disrupts its translation. Thus, the decrease in repression of HapR translation will only occur when the HapR already present in cells has degraded.

Figure 3.10 Plots showing the characteristics of different Qrrs. **A)** Graphical depiction of the Qrr/HapR testing circuit. Both states of the circuit are shown for better clarity. Upper diagram: HapR expression is constitutive and thus the RFP expression is repressed by HapR binding to the pAphA. Lower diagram: Addition of arabinose starts expression of Qrr which disrupts translation of HapR. In effect the RFP can be expressed from the pAphA. **B)** and **C)** Comparison of RFP fluorescence/OD₆₀₀ of cultures harbouring the Qrr/HapR constructs with different Qrrs in different arabinose concentrations after 6h of cultures. The advantage of Qrr4 over the rest is clear. **D)** Plot showing the RFP fluorescence/OD₆₀₀ in time of cultures

expressing Qrr4. The constitutive expression of HapR without any presence of Qrr is shown for comparison.

Figure 3.11 Predicted structures of the Qrrs.

Since HapR can both act as repressor and activator it should be possible to use it as a single protein demultiplexer circuit. In such a circuit one input signal is processed into two distinct output signals. To achieve that GFP under control of the pLux promoter have been added to the circuit used to test the HapR repression efficacy (Figure 3.12). As was mentioned before the pLux promoter is activated by presence of HapR rather than repressed like the pAphA. This means that this new system by principle should produce RFP only when there is no arabinose in the medium and produce GFP when arabinose is available whereas switching off RFP production at the same time. Figure 3.13 shows the levels of GFP and RFP divided by cultures OD_{600} after 6 hours. As one can see without arabinose the system behaves as predicted, the RFP level is high and GFP is not produced. However, after addition of arabinose to activation levels (in this case 0.02%, limited by negative final OD impact of HapR overexpression) the not very effective repression achieved by HapR coupled by low efficacy of activation of pLux by HapR results in a state where both RFP (however lowered) and GFP are present in the cells instead of the expected profile. This would limit the use of such multiplexer to very specific situations

where the repression of the protein expressed constitutively (i.e. from pAphA) does not have to be complete. Additionally, the low activation of pLux by HapR would limit the overall usability of the system for protein expression.

Figure 3.12 Graphical depiction of the demultiplexer. Both states of the circuit are shown for better clarity. Upper diagram: there is no HapR expression due to lack of arabinose in the medium and as a result the RFP expression is not repressed by HapR and GFP is absent due to lack of activation of pLux. Lower diagram: Addition of arabinose starts expression of HapR which represses the RFP and activates GFP expression from pLux at the same time.

Figure 3.13 Plot showing the GFP/OD₆₀₀ and RFP/OD₆₀₀ of cultures with the multiplexer after 6h. Addition of arabinose should change the state of the expression to the opposite one, that is from high RFP and low GFP to low RFP and high GFP.

In summary, the key points of the examined HapR/Qrr based inverter would be:

- a) The HapR is able to repress pAhpA in *E. coli*, but not effective enough to be considered a good repressor.
- b) Overexpression of HapR can inhibit growth of the culture considerably. However, good repression is achieved with HapR levels that do not impose this effect
- c) HapR is able to induce expression from pLux in only limited capacity in *E. coli*
- d) Qrrs can be used as effective inhibitors of HapR expression, with notable example of Qrr number 4

All the above points made it obvious that adaption of this system for the purpose of the *V. cholerae* detecting circuit would require serious optimisation work and still may end up in a failure. Because of this it was decided not to pursue further studies on the usage of the HapR/Qrr based inverter.

3.3.3 Other TetR type repressor based inverters

Since HapR proved unable to deliver anticipated results, it was decided to assess other TetR type repressors (of which type HapR is part of) for feasibility of use in the sensor system [53]. The TetR family of repressors takes its name from TetR, repressor protein involved in tetracycline resistance in bacteria. TetR type repressors can be found in many different species of bacteria, also in *E. coli*. However, it would be extremely beneficial to use one from a genetically distant bacterium in the designed system, as this would prevent any unwanted interference with the basic genomic expression of *E. coli*. Voigt et al. have characterised a number of TetR like repressors together with their respective promoters [164]. They used IPTG induced promoter to show the level of repression of output protein with different expression levels of the given TetR type protein (much like it was done for this work in the previous subchapter). The results for some of the tested proteins can be seen in Figure 3.15. There are

a few things of note in that Figure. First, it shows why TetR itself is a very popular repressor (and inducible one at the same time) by having high expression and repression capability. Another thing of note would be that some of the other of the repressors have very similar, sometimes even better (by better higher constitutively expression and high repression is meant), shape of the repression curve to that of TetR. One of such proteins would be PhlF. Final interesting thing shown in this graph is the shaded area in some of the plots, representing the expression level of the repressor that is toxic to the cell, much like HapR was found to be in this work.

Figure 3.14 Set of plots showing characteristics of different repressors from the TetR family. Adapted from [164] with permission.

With all the above in consideration it has to be noted that all of them bear another huge disadvantage that effectively bar use of any of them in the designed system. This fault becomes obvious when one will take into consideration the low expression level of truncated pQrr4 (the actuator module). This means that the potential inverter (repressor) has to repress its respective promoter when its concentration in the cell is very low. Unfortunately, none of the tested repressors showed this very important quality. Because of this it was decided not to proceed with follow-up experiments involving other TetR family repressors in this work.

3.3.4 CRISPR based inverter

Because CRISPRi gives the flexibility to design gRNA to repress different promoters of different strengths and has very high repressing efficiency, it was decided to try using a CRISPRi based inverter for the inverting module. Furthermore, CRISPRi also gives one the ability to readily generate a library of inverters using promoters of very different strengths. This would enable one to match the required sensor output with a promoter with a suitable strength.

Figure 3.15 Graphical depiction of the CRISPRi testing circuit. The gRNA and dCas9 are both under inducible promoters and by changing the levels of the respective promoters the repression level of CRISPRi target promoter and consequently GFP expression can be manipulated. Reproduced from [167] with permission.

To create the desired inverter, it is first required to establish which of the two components of the CRISPRi system (i.e., dCas9 and gRNA) should be

placed under the control of tpQrr4 promoter. It could be either the dCas9 or the gRNA, or even both. The other component would be expressed constitutively. The criterion for this choice was – by manipulating the cellular levels of which of the two components, a high level of repression of the target promoter at low induction gRNA/dCas9 level can be achieved. To elucidate the choice, eight inverters using a set of four promoters from the widely used Anderson promoter library were designed and constructed [171] (Figure 3.15). The promoters that have been chosen have two main qualities – they do not differ much between each other in terms of sequence but they have different promoter strength. For each of the promoters two distinct gRNAs were designed - one targeting -10 region of the promoter and binding to the non-template strand and one targeting -35 region of the promoter and binding to the template strand. To study the characteristics of the inverters under different expression level of dCas9 and gRNA, dCas9 and gRNA have been placed under ATc (anhydrotetracycline) and IPTG (Isopropyl β -D-1-thiogalactopyranoside) inducible promoters respectively (Figure 3.15).

Table 3.1 Table showing the relative strengths of the Anderson promoters used in this study.

Promoter designation	Relative strength [171]
J23101	0.70
J23109	0.04
J23110	0.33
J23115	0.15

Figure 3.16 Heat maps representing the repression efficiency of a given expression levels combination of gRNA and dCas9. Increasing level of a given inducer increases the level of either the gRNA or dCas9. The repression level is normalized where the expression from a given promoter with no inducers added is 1. The -10 or -35 in the plot designation relates to the part of the promoter that is the target of the promoter, whereas the three last digits designate the promoter number (see table 3.1). Reproduced from [167] with permission.

After examining all eight CRISPRi constructs, eight heat map type plots were generated, one for each construct (Figure 3.16). In these heat maps it can be seen that very similar level of expression is achieved for all dCas9 levels at a given gRNA level, implying that the inverter is not sensitive to dCas9 level

variations. On the other hand, for a given dCas9 level a range of repression levels are achieved for different gRNA levels. Based on this observation, it was decided that the gRNA will be produced under tpQrr4, whereas dCas9 will be placed under a weak constitutive promoter. This has the added advantage of preventing dCas9 expression level going above toxicity threshold (28). Figure 3.17A,B shows the transition curves of promoters targeted at -10 and -35 site respectively. As can be seen in these graphs, a whole range of inverters with different final repression levels and repression profiles has been created. Since tpQrr4 is a weak promoter, it is necessary for the inverter to have a gRNA - promoter pair where the promoter strength is high so as to amplify the output signal (GFP) but at the same time the promoter needs to be very strongly repressed by low gRNA levels which is expressed by tpQrr4. Both of these conditions have been met by inverter with JS23115 promoter with its respective gRNAs (Figure 3.18).

To validate the hypothesis that the inverter will behave in a similar manner if the pLlacO-1 inducible promoter driving dCas9 expression is replaced by a weak constitutive promoter, a modified version of the construct in which GabDP2 constitutive promoter drives dCas9 expression was prepared. As shown in Figure 3.17C, it is clear that upon adding IPTG in concentration of $> 25\mu\text{M}$, the expression of GFP is sharply repressed to nearly non-detectable levels. Further, compared to wild type MG1655 *E. coli* there is no change of growth in the investigated timeframe. This implies that the inverter system expressing dCas9 under GabDP2 and gRNA does not introduce significant metabolic burden to the host.

Figure 3.17 Plots showing repression curves for -10 (A) and -35 (B) targeting inverters with increasing gRNA (IPTG) level. dCas9 has been expressed with 0.5 nM ATc. Descriptions of curves as in Figure 3.17. Reproduced from [167] with permission. C) Repression curve for the -10 site targeting gRNA of JS23115 promoter in different gRNA (IPTG) concentrations. dCas9 is been constitutively expressed under GabDP2 promoter. Reproduced from [167] with permission.

3.3.5 Conclusion

After thorough investigation of possible designs, it was found that TetR family repressors do not meet the two main design goals for the potential inverting module – they do not repress when in low concentration and they become toxic to the cells when in high concentrations, even if their “middle ground” shows to be very usable in other applications. To conclude, the CRISPRi based inverter showed the most promise for use in the designed inverter module. As was mentioned before, since the combination of JS23115 promoter with the gRNA targeting its -10 site shows very high repression efficiency even in low gRNA levels combined with enough promoter strength, it is the one chosen as the repressor for the inverter module.

3.4 Characterization of the sensor module

3.4.1 Combinatorial design of sensor constructs

The sensor module of the system basically comprises three proteins – CqsS (the membrane CAI-1 detecting protein), LuxU (the phosphorelay protein) and LuxO (the phosphorylation dependent transcription factor). The tpQrr4 is also part of the sensor module as it is activated by LuxOp. Since all the three proteins can be expressed independently from each other (i.e. using a separate promoter and RBS) but the effects of their expression can be measured only if all three of them are expressed, what is the optimal expression level of each of them that will produce the desired expression from tpQrr4? There are potentially thousands of combinations of promoters and RBS that can be used here (in case one would limit oneself only to Andersons promoter list and iGEM RBS list [171, 172]). However, it should be possible to “scan” the possible design space to find out how expression levels of different proteins influence the final expression from the tpQrr4. The goal here would be finding a construct where expression levels of the sensor proteins give us the highest possible expression from the tpQrr4. This comes from the hypothesis that higher expression from tpQrr4 will produce more gRNA in the final construct in effect producing more repressed GFP expression when no *V. cholerae* will be present.

Figure 3.18 Graphical depiction of the CRISPRi testing circuit. The constitutively expressed sensor proteins stay all phosphorylated making the truncated Qrr4 promoter constitutively ON. This ensures constitutive GFP expression.

To do this a double plasmid system has been constructed (Figure 3.18). The first plasmid comprised GFP under the control of truncated pQrr4 promoter. This made it express GFP when phosphorylated LuxO was present in the cell. The second plasmid comprised CqsS, LuxU and LuxO all constitutively expressed

by strong constitutive promoter p66. To alter the expression level of each of them different RBS for each protein for each of the combinatorial designs were chosen (by combinatorial design it is here understood a set of designs with the same function but slightly different expression levels of constituents). These different RBS and respective designs are summed up in Table 3.2. It was opted for the lowest strength RBS for CqsS in all of the constructs, because of the well-known negative impact of membrane proteins overexpression on cell growth [173]. After preparing all seven plasmids (one with GFP one and six with sensor proteins) they were transformed into *E. coli* cells and characterized.

Table 3.2 Different combinatorial designs (L, ML, MH, H refer to Low, Medium Low, Medium High and High strength RBS respectively).

	RBS [174]		
Design name	CqsS	LuxU	LuxO
LLL	BBa_B0033 (Relative strength 0.01)	BBa_B0033 (Relative strength 0.01)	BBa_B0033 (Relative strength 0.01)
LLH	BBa_B0033 (Relative strength 0.01)	BBa_B0033 (Relative strength 0.01)	BBa_B0034 (Relative strength 1)
LMLML	BBa_B0033 (Relative strength 0.01)	BBa_B0031 (Relative strength 0.07)	BBa_B0031 (Relative strength 0.07)
LMLMH	BBa_B0033 (Relative strength 0.01)	BBa_B0031 (Relative strength 0.07)	BBa_B0032 (Relative strength 0.3)
LMHML	BBa_B0033 (Relative strength 0.01)	BBa_B0032 (Relative strength 0.3)	BBa_B0031 (Relative strength 0.07)
LMHMH	BBa_B0033 (Relative strength 0.01)	BBa_B0032 (Relative strength 0.3)	BBa_B0032 (Relative strength 0.3)

The comparison of final expression levels from different sensors is summed up on Figure 3.19. It shows that there seems to be a negative correlation between the level of expression of sensor proteins and GFP. The only outlier seems to be the LMLMH construct. However, no clear relationships between expression levels of CqsS, LuxU and LuxO and final GFP expression could be found. The next logical step would be expanding the tested space by adding more combinations of RBS, but this would require long and tedious testing and still would not guarantee finding the right relationships. In this case, since it would not be practical to screen and prepare all the required constructs it was decided to prepare an *in silico* mathematical model that would describe the phosphorylation cascade in the cell. This way the whole possible space of different expression levels can be analyzed *in silico* without having to construct each and every variant of the circuit. Based on this analysis an optimal expression level should be established.

Figure 3.19 Final expression level of different constitutive sensors. There seems to be a decreasing trend in the final expression with increasing expression level of the proteins, with the exception of LMLMH. Readings taken after 6 hours of culture.

3.4.2 Model of the phosphorylation cascade

The model describes three *in vivo* processes by means of ODE (ordinary differential equations). Those processes were: i) transcription and translation of genes, ii) phosphorylation cascade and iii) CRISPRi based repression of the receiver promoter (since the gRNA is produced using tpQrr4 and the design goal is to produce gRNA on levels that would ensure distinctive ON and OFF states of the sensor).

ODE describing the transcription and translation of genes are shown below:

$$\frac{d[mRNA]}{dt} = v_{mRNA} - \gamma_{mRNA}[mRNA] \quad \text{Eq. 1}$$

$$\frac{d[mRNA]}{dt} = V_0 + \frac{V_{\max} \times [L]^n}{[L]^n + K_D^n} - \gamma_{mRNA} \times [mRNA] \quad \text{Eq. 2}$$

$$\frac{d[protein]}{dt} = \alpha_{protein}[mRNA] - \gamma_{protein}[protein] \quad \text{Eq. 3}$$

where:

v_{mRNA} - promoter-dependent constitutive transcription rate,

$\alpha_{protein}$ - ribosome binding site (RBS)-dependent translational rate constant,

γ_{mRNA} and $\gamma_{protein}$ - mRNA and protein degradation rate constant, respectively.

L - ligand concentration

V_0 and $V_{\max}$ - basal and maximum transcription rate of the promoter.

K_D - ligand concentration which produces half occupation

n - the hill coefficient.

Eq. 1 is a linear function describing constitutive transcription of genes, Eq.2 describes mRNA transcription from inducible promoter (using Hill equation) and finally Eq.3 describes mRNA translation.

The equations describing the phosphorylation cascade are shown below. It is assumed that the autophosphorylation of CqsS is irreversible. It is also assumed that the CqsS and CAI-1 may not be binding in 1:1 ratio ($n1 \neq 1$, Eq. 4-5). Also, ultrasensitivity was used to describe the phosphorylation and dephosphorylation as shown in Eq. 6-9 [175].

$$\frac{d[CqsS - CAI1]}{dt} = k_{f1} \times [CqsS] \times [CAI]^{n1} - k_{r1} \times [CqsS - CAI1] \quad \text{Eq. 4}$$

$$\frac{d[CqsS_{pp}]}{dt} = k_{pp} \times [CqsS] \quad \text{Eq. 5}$$

$$\frac{d[LuxU]}{dt} = -k_{f21} \times [LuxU] \times [CqsS_{pp}] + k_{r21} \times [LuxU - CqsS_{pp}] + k_{cat22} \times [LuxU_p - CqsS] \quad \text{Eq. 6}$$

$$\frac{d[LuxU - CqsS_{pp}]}{dt} = k_{f21} \times [LuxU] \times [CqsS_{pp}] - (k_{r21} + k_{cat21}) \times [LuxU - CqsS_{pp}] \quad \text{Eq. 7}$$

$$\frac{d[LuxU_p]}{dt} = -k_{f22} \times [LuxU_p] \times [CqsS] + k_{r22} \times [LuxU_p - CqsS] + k_{cat21} \times [LuxU - CqsS_{pp}] \quad \text{Eq. 8}$$

$$\frac{d[LuxU_p - CqsS]}{dt} = k_{f22} \times [LuxU_p] \times [CqsS] - (k_{r22} + k_{cat22}) \times [LuxU_p - CqsS] \quad \text{Eq. 9}$$

Where:

CqsS and LuxU - free CqsS and LuxU protein which act as the phosphatases in this cascade

CqsS_{pp} and LuxU_p - autophosphorylated CqsS and phosphorylated LuxU— acting as the kinases of this cascade

k_{f1} , k_{f21} and k_{f22} - association constants of enzyme-substrate complex formation

k_{r1} , k_{r21} and k_{r22} - disassociation constant of complex

$n1$ - binding coefficient between CAI-1 and CqsS

k_{pp} - auto-phosphorylation rate constant

k_{cat21} and k_{cat22} - catalytic rate constant

Additionally, total concentration of each protein has been described using the equations below:

$$[CqsS_{TOT}] = [CqsS] + [CqsS_{pp}] + [CqsS - CAI1] + [CqsS - LuxU_p] + [CqsS_{pp} - LuxU] \quad \text{Eq. 10}$$

$$[LuxU_{TOT}] = [LuxU] + [LuxU_p] + [CqsS - LuxU_p] + [CqsS_{pp} - LuxU] + [LuxU - LuxO_p] + [LuxU_p - LuxO] \quad \text{Eq. 11}$$

$$[LuxO_{TOT}] = [LuxO] + [LuxO_p] + [LuxU - LuxO_p] + [LuxU_p - LuxO] \quad \text{Eq. 12}$$

Finally, description of the CRISPRi repression has been prepared. Formation of gRNA-dCas9 complex has been described using reversible mass action law (Eq. 13). Then, assumption was that the CRISPRi interference can be modelled with Hill equation (Eq. 14), which mediates the expression of reporter (GFP, Eq. 15).

$$\frac{d[gRNA - dCas9]}{dt} = k_{f41}[gRNA] \times [dCas9] - k_{r41}[gRNA - dCas9] \quad \text{Eq. 13}$$

$$\frac{d[mRNA_{GFP}]}{dt} = \frac{V_{max} \times K_D^n}{[gRNA - dCas9]^n + K_D^n} - \gamma_{mRNA} \times [mRNA_{GFP}] \quad \text{Eq. 14}$$

$$\frac{d[GFP]}{dt} = \alpha_{protein}[mRNA_{GFP}] - \gamma_{protein}[GFP] \quad \text{Eq. 15}$$

Where:

k_{f41} and k_{r41} - association and disassociation constant of gRNA-dCas9 ribonucleoprotein γ_{mRNA} and $\gamma_{protein}$ - mRNA and protein degradation rate constant, respectively

K_D - ligand concentration which produces half occupation, and n refer to the hill coefficient

Figure 3.20 Parameter estimation of constitutive/inducible gene expression using the least squares method. **A)** constitutive transcription determined by characterizing fifteen promoters; **B)** translation determined by characterizing eight RBSes; **C)** inducible transcription determined by characterizing three inducible promoters, each of them was induced with inducer in eight different concentrations. x-axis in **(A)** and **(B)** shows the first two hours in the exponential phase [min], and in **(C)** it refers to the first three hours in the exponential phase [min] with an empirical estimation of one hour where transcriptional factors accumulate and activate inducible promoter. y-axis in **(A)** and **(B)** shows the RFP

fluorescence / OD₆₀₀ [a.u.] and in (C) is inducer concentrations. Initial condition for all simulation was read from experimental data at 30 mins. Reproduced from [167] with permission.

Similarly, as for the cascade total protein concentrations have been described with equations below:

$$[gRNA_{TOT}] = [gRNA] + [gRNA - dCas9] \quad \text{Eq. 16}$$

$$[dCas9_{TOT}] = [dCas9] + [gRNA - dCas9] \quad \text{Eq. 17}$$

After all the above processes has been described parameter estimation was performed. A simultaneous approach, which searches the optimal parameter combination that minimizes the deviation of simulated prediction from the experimental data was chosen [176]. This was done by employing non-linear least square curve fitting tools. After the parameter was established, another set of data was used for parameter validation.

Figure 3.21 Parameter estimation of the (de)phosphorylation of CqsS (subprocess 1 (SP1)) and LuxU (sub-process 2 (SP2)). A) Parameter estimation of SP1. At a given CqsS concentration of 2μM, autophosphorylation of CqsS was examined by adding different CAI-1 concentrations. B) Parameter estimation of CAI-1 dependent phosphorylation in SP2. At a given CqsS concentration 2μM, eight different CAI-1 concentrations were applied to affect the autophosphorylation of CqsS (SP1). After 10 mins, phosphorylation of LuxU occurred and was examined after 30s. C) Validating the parameters estimated in SP1 and SP2 by CAI-

1 independent dephosphorylation in SP2. At a given CqsS concentration of 4 μ M, without CAI-1, CqsS and LuxU were phosphorylated. After 5 mins, phosphorylated LuxU was rinsed out and used in a new system with a different CAI-1 and CqsS concentration. Reduction of phosphorylated LuxU was examined during 8 mins. All experimental data are derived from study [60]. Reproduced from [167] with permission.

For initial parameter estimation for transcription and translation unpublished data characterizing a set of constitutive and inducible promoters, as well as RBS were used (Figure 3.20). For estimating the parameters of the phosphorylation cascade data provided from one of the in vitro studies of the cascade have been used [60] (Figure 3.21). For parametrization of CRISPRi equations data from the CRISPRi matrixes and tpQrr4 characterization were used.

Figure 3.22 Parameter estimation of the tpQrr4 promoter. In genetic circuits: (p15A) pBAD-LuxOt-tpQrr4-GFP (Figure 3.3), A) parameter estimation was performed using experimental data measured using four randomly selected arabinose concentrations: 0.002% (w/v), 0.016%, 0.031% and 0.063%. B) Parameters were validated using experiment data measured on the other four arabinose concentrations: 0.001%, 0.004%, 0.008% and 0.125%. Initial condition for all simulation was read from experimental data at 30 mins. Reproduced from [167] with permission.

To validate the parameters of the sensor module, a genetic circuit was built which comprises CqsS-LuxU-LuxO and tpQrr4 promoter to drive the expression of GFP (Figure 3.23A). Variations of this genetic circuit were constructed by changing the promoters transcribing LuxO (pLac-O1 or pBAD) or LuxO RBS (weak or strong). Figure 3.23B,C show that the model is able to reproduce the

sensor behavior when the RBS of LuxO gene is changed and various IPTG concentrations are added (r-square: 0.82 and 0.89 in B and C, respectively).

Figure 3.23 Parameter validation for sensor module. A) Genetic circuits used in validating sensor parameters. B) and C) validation with different experimental settings: having r-square = 0.82 in (B), r-square = 0.89 in (C). Initial conditions for all simulation were read from experimental data at 30 mins. Reproduced from [167] with permission.

Figure 3.24 Parameter estimation and validation for inverter and reporter. A) Parameter estimation was performed using genetic circuits shown here, J23115 (with site -10 targeted) is selected to be the CRISPRi repressive promoter. Over the twenty-eight combinations from seven IPTG concentrations and four ATC concentrations, only the relative GFP at 2 hours is shown in (A). B) Parameter validation was performed using genetic circuits in which dCas9 is transcribed by GabDP2 constitutive promoter. Reproduced from [167] with permission.

Parameters for inverter and reporter were estimated using genetic circuits shown in Figure 3.24B. First, parameters describing pLlac-O1 and pTet promoters were derived using genetic circuits shown in Figure 3.24B. Next, parameters used in CRISPRi-based inverter were estimated using experimental data from twenty-eight combinations of gRNA and dCas9, as described earlier (Figure 3.16). After estimating the parameters, the derived values were validated using experimental data from the circuit where dCas9 was constitutively transcribed by GabDP2 promoter (Figure 3.17).

Figure 3.25 Sensitivity analysis of the model. Highlighted are the parameters which can be potentially changed in the physical circuit. Reproduced from [167] with permission.

To study which model parameters are the key factors that affect the model output significantly, sensitivity analysis (SA) implemented in Simbiology toolbox of Matlab (Mathworks) was performed. The major task of carrying out the SA is to find out which parameter(s) affect the gRNA production and can be physically changed (like promoters or RBS). Figure 3.25 shows the sensitivity indices (SI) of the model parameters. An empirical threshold ($r=0.6$) is applied

to discriminate the significance from the insignificant model parameters. One can note that the transcription of LuxO protein critically affects gRNA production and, subsequently, the GFP production. Because of this an inducible promoter was chosen for the LuxO transcription as this would give an option for easy change of its expression level. Therefore, the ODE of LuxO transcription has been updated accordingly with the following equation:

$$\frac{d[LuxO_{mRNA}]}{dt} = V_0^3 + \frac{V_{\max}^3 \times [IPTG]^{n_4}}{[IPTG]^{n_4} + K_{D3}^{n_4}} - \gamma_{mRNA}[LuxO_{mRNA}] \quad \text{Eq. 18}$$

Where the p_{lac}-O1 promoter is the selected one.

After constructing and validating the model conclusions has been drawn on how the sensor part of the system should be designed. The level of gRNA produced within the input dynamic range of the CRISPRi-based JS23115 inverter has been determined first. Here, input dynamic range refers to the range of input signal (gRNA) over which JS23115 inverter could respond. From previous tests it is known that the reporter GFP expression reduces from 100% to 10%, when IPTG concentration increases from 0 μ M to 25 μ M. As p_{lac}-O1 promoter drives the expression of gRNA, this 10% reporter expression level corresponds to a 90% repression caused by a gRNA level induced by 25 μ M IPTG. Using the model, the dynamics of gRNA induced by IPTG have been simulated. The result suggests that the dynamic range of repression corresponds to 0-0.5 μ M of gRNA (Figure 3.26). To fine-tune the sensor circuit design to achieve the expected gRNA level, as was said before, a genetic circuit in which LuxO is transcribed by an IPTG-inducible promoter (p_{lac}-O1) has been constructed to study and determine the optimal expression level of LuxO by varying IPTG concentration (This construct was used in the study under “Integration of the sensing system” section).

A number of simulations using the model has been run to gain insights into what would the optimal amount (ratio) of LuxO, LuxU and CqsS be. LuxO has been studied first. Phosphorylated LuxO (LuxOp) activates the tpQrr4

promoter which drives the gRNA expression. It was found that the percentage of LuxOp with respect to total LuxO (LuxO_{TOT}) correlates to the amount of gRNA presence (r-square > 0.96, Figure 3.26B). This correlation suggests that one can tune the gRNA level by adjusting the ratio between LuxOp and LuxO_{TOT}. It was also noted that LuxO_{TOT} induced by different amounts of IPTG transform 60-80% of LuxO_{TOT} into LuxOp. This amount of LuxOp produces enough gRNA because the saturated gRNA level is higher than the gRNA needed for full repression. Based on this simulation result, hypothesis was drawn that the sensor can work properly with different amounts of LuxO_{TOT}.

The model also suggests that phosphorylated LuxU (LuxUp) significantly affects LuxOp level. Figure 3.26C shows that only a small amount of LuxUp is needed to tune the LuxOp and gRNA level. LuxUp amount is determined by LuxUTOT amount and its phosphorylation/dephosphorylation. The model shows that autophosphorylated CqsS (CqsSpp) enables up to 99% of LuxU_{TOT} to be transformed into LuxUp (Figure 3.26C). Together with the small amount of LuxUp needed, it can be inferred that weak expression of LuxU would keep the sensor working properly.

CqsS is a membrane protein and is difficult to express in the genetically engineered *E. coli* [173]. Since only a small amount of CqsSpp is required to transform 99% of Lux-U_{TOT} into LuxUp (Figure 3.26C), a weak expression of CqsS for the sensor circuits would be sufficient and desirable. The model shows that with a low level of CqsS_{TOT}, the sensor detects the presence of CAI-1 and prevents the formation of CqsSpp. As a result, gRNA production would be repressed (Figure 3.26D) which would then deactivate the inverter and allow the reporter to produce maximum amount of GFP upon the detection of CAI-1 (Figure 3.26E).

Figure 3.26 Simulation of sensor module aims to advise the optimal circuit design A) Simulated gRNA production to fulfil the needs of the inverter. In CRISPRi-based Js23115 inverter gRNA is transcribed by IPTG induced pLacI-O1 promoter. Figure 3.17 shows that the input dynamic range (90% repression) of JS23115 inverter is 0 - 25 μM IPTG. Simulation results suggest this input dynamic range corresponds to a gRNA production of 0-0.5 μM per OD600. B) Effects of LuxO (total and phosphorylated) on gRNA production. Proportion (% LuxOp) of phosphorylated LuxO (LuxOp) in total LuxO protein (LuxO_{TOT}) and gRNA production is simulated in sensor circuit which comprises a strong expression of CqsS, increasing expression of LuxU and IPTG-induced expression of LuxO. LuxO_{TOT} is induced by 0, 3, 12, 100 μM IPTG respectively (from left to right). For all these four IPTG concentrations, phosphorylated LuxU (LuxUp) controls % LuxOp and gRNA production. C) Effect of LuxU (total and phosphorylated) on gRNA production. Proportion (% LuxUp) of phosphorylated LuxU (LuxUp) in total LuxU protein (LuxU_{TOT}) and proportion (% LuxU) of unphosphorylated LuxU (LuxU) in total LuxU protein is simulated in sensor circuit which comprises an increasing expression of CqsS, strong expression of LuxU and 0 μM IPTG-induced expression of LuxO. Simulation result shows that both proportions are controlled by autophosphorylated CqsS (CqsSpp) and the turn-over of LuxU_{TOT} into LuxUp could reach 99%. D) Effect of CAI-1 on gRNA production is simulated in sensor circuits which comprises weak expression of CqsS and LuxU and 0 μM IPTG-induced expression of LuxO. Simulation result shows that CAI-1 controls the proportion (% CqsSpp) of CqsSpp in total CqsS (CqsS_{TOT}) and gRNA production. The model also predicts the response of GFP after simulation the inverter and reporter module. Reproduced from [167] with permission.

Hence, the model proposes an optimal circuit design for the sensor module, which comprises weak constitutive expression of CqsS and LuxU, together with inducible expression of LuxO to retain the ability to change LuxO levels if needed. There were two reasons to do this. One is that the LuxO, according to the model, is the key protein in the whole system and its level will have very high impact on the final system performance. The second reason is that by turning LuxO expression ON and OFF impact of the sensor has on the inverter can be investigated.

3.5 Integration of the sensing system

Based on the design suggested by the model a full system circuit which comprises the sensor layered with the inverter and reporter was constructed. A double plasmid system showed in Figure 3.27 has been prepared. The final system was studied using *E. coli* and *V. cholerae* overnight supernatants. *E. coli* supernatant is CAI-1 free and *V. cholerae* supernatant is reported to contain 1.25 μM of CAI-1 when *V. cholerae* is cultured to high density [31]. Also, this is the concentration found to shift *V. cholerae* cells from low to high density state [55].

Performed tests show that when the full system is grown in increasing concentrations of the supernatant (5, 30, 50 and 90 %) in a range of IPTG concentrations (from 0 to 500 μM), the highest achievable difference between final GFP/OD600 of cultures grown in *E. coli* and *V. cholerae* supernatant is 2.5 fold (90% supernatant, 0 μM IPTG) (see Figure 3.28A). Results show that increasing concentrations of supernatant increases the fold difference between the final GFP/OD600 of the cultures with *V. cholerae* supernatant and the cultures with *E. coli* supernatant. This is most probably the effect of increasing CAI-1 concentration in the medium and hence decreasing intracellular level of phosphorylated LuxO. On the other hand, when the intracellular level of LuxO is increased with addition of IPTG, the final level of GFP/OD600 decreases for both the cultures with *E. coli* and *V. cholerae* supernatant respectively. However, despite the fact that the decrease in the former case is lesser than in the latter, resulting decrease of fold difference with increasing IPTG concentration is very

small (Figure 3.28B, compare Figure 3.28A). These results are also in line with predictions from the model, when it is used to predict fold difference (Figure 3.28C)

Figure 3.27 Graphical depiction of the final sensor circuit. A) In this diagram double plasmid sensor circuit in low *V. cholerae* cell density state is shown. CqsS, LuxU and LuxO are constitutively expressed and phosphorylated. Phosphorylated LuxO activates Qrr4 promoter which expresses gRNA. This gRNA joins constitutively expressed dCas9 and they together repress GFP expression from a constitutive promoter. B) In this diagram the same circuit is shown but in high *V. cholerae* cell density state. CAI-1 dephosphorylates CqsS which ultimately dephosphorylates LuxO. Dephosphorylated LuxO does not activate the Qrr4 promoter preventing gRNA expression. With no gRNA present GFP can be expressed efficiently. A) and B) In blue are parts of the sensor module, in red are parts of the inverter module and finally in green is the receiver module. Text in circles denotes respective plasmid origin. Reproduced from [167] with permission.

Figure 3.28 Full System Integration A) Bar graph showing the inverted sensor behaviour following increasing IPTG (LuxO) and supernatant concentrations. The fold difference in GFP/OD600 after 6 hours between cultures with *V. cholerae* supernatant and *E. coli* supernatant added increases with increasing supernatant percentage. The same fold difference does not change substantially with increasing IPTG (LuxO) level. B) Graph showing final level of GFP/OD600 of cultures with different IPTG levels and with 90% addition of supernatants. Although the fluorescence levels differ between different IPTG levels the fold difference is maintained for all the IPTG concentrations. C) Prediction of the model for cultures as in B). The model predicts the shape of the curves; however, it indicates higher fold difference between the two supernatants. D) and E) Graphs showing characteristics of the tested system in time. Both plots show cultures with 90% supernatant content, D) is with no IPTG added and E) is with 500 μM IPTG. As far as final fold difference in GFP/OD600 is very similar for both, the final levels of GFP/OD600 differ substantially between these two cases. F) Result of in silico simulation of cultures with 90% *V. cholerae* and *E. coli* supernatants with no IPTG added. The model accurately predicts behaviour of the system (compare with 5D) after the difference between the two cultures becomes visible – it does not model the lag phase seen in the actual experiment. Reproduced from [167] with permission.

This suggests that by increasing LuxO level one can decrease the basal (uninduced) GFP expression, as the higher level of phosphorylated LuxO produces more gRNA. However, at the same time the system produces less GFP upon CAI-1 detection. This may be because the higher amount of LuxO proteins could no longer be efficiently dephosphorylated by the given amount of CqsS, as suggested by the model analysis. As a result, the final gRNA level in these two cases would be higher than when IPTG was absent.

Further, when IPTG is absent, the baseline response of the sensor to *E. coli* supernatant looks almost like an induction curve (Figure 3.28D). This implies that the false positive rate of this sensor with no IPTG would be very high. On the other hand, although the absolute GFP/OD600 reading is lower with IPTG, the baseline response of the sensor to *E. coli* supernatant looks much more stable (Figure 3.28E). This suggests that the sensor with IPTG added would be more reliable.

To confirm the response of the system to the *V. cholerae* supernatant, a variant of the circuit was constructed without the inverter module and with GFP being expressed directly by tpQrr4 (similar to what was done in combinatorial study but the p66 promoter for LuxO has been exchanged with pLacO₁). As expected, GFP/OD-600 reading of cultures of the said construct with *V. cholerae* supernatant added (50% of the medium) and without IPTG added are about 15% lower than those with *E. coli* supernatant (Data not shown). This difference is likely due to the result of lower LuxOp level in the cell upon CAI-1 detection. After addition of IPTG to the medium (500 μ M), the difference is lowered to around 10% with slight increase in absolute expression (effect of overall higher LuxOp level). However, this expression is much lower than in the system with inverter as the inverter doubles as an amplifier for the weak tpQrr4. Direct final GFP/OD600 reading comparison of the two constructs (inverted and non-inverted, with no IPTG and supernatant added to the culture) shows that the amplification ratio of the inverter module is around 30 (see Figure 3.29).

Figure 3.29 The comparison of systems with and without CRISPRi inverter present. The cultures were grown in LB without supernatants. No IPTG has been added to either of them. Reproduced from [167] with permission.

3.6 Sensor selectivity study

To check if the sensor is sensitive only to CAI-1 containing *V. cholerae* supernatant it was decided to test the sensor against one additional *E. coli* strain (Nissle 1917), three additional *V. cholerae* strains (A1522 producing rugose colonies, El Tor and El Tor Δ HapR) and one strain of each of the following: *Bacillus subtilis*, *Pseudomonas putida*, *Pseudomonas aeruginosa* and *Lactobacillus rhamnosus*. Tests were conducted in 90% of supernatant content and with 0 and 500 μ M IPTG.

As can be seen on the Figure 3.30, the other *E. coli* strain is unable to activate the system and so is *L. rhamnosus*. The first surprise is the fact that the system is not activated by *V. cholerae* El Tor, which is the infectious strain. This could be explained by the fact that the isolate of El Tor obtained by the author for this research was isolated at unknown point in the past and its ability to produce CAI-1 has been lost due to high number of passages done with it. The next surprising fact is that the sensor is somewhat activated by *B. subtilis* and *P. aeruginosa* and highly activated by *P. putida*. This may be due to several reasons:

- a) The aforementioned bacteria are producing some kind of CAI-1 analogue that is detected by CqsS. Since it was never looked for in those strains it is possible that they are producing such analogues for communication or some other reason. That would mean that CAI-1 is not as *V. cholerae* specific as was first thought. This notion is reinforced by the fact that both *Pseudomonas* and *B. subtilis* are found to be parts of human microbiome and it could be beneficial for *V. cholerae* to have extra means to detect the host via host microbiome detection.
- b) There can be some interaction between LuxU or/and LuxO and *E. coli* two component systems. If those systems are part of quorum sensing mechanisms, known or unknown, it could explain the fact of detection of other than *V. cholerae* bacteria.
- c) Due to the fact that CqsS is not natural part of *E. coli* there can be some structural problem with its folding or placement in the membrane. This could make it less specific to CAI-1 like molecules.

However, since the sensor can readily differentiate between *V. cholerae* and *E. coli* it was decided that it can be used for further studies with the specificity problem to be solved in the near future.

Figure 3.30 Specificity of the biosensor. A) Comparison of fold difference between cultures done in *E. coli* MG1655 and other species supernatants, both without and with IPTG added to the medium. B) Changes in GFP/OD₆₀₀ of cultures in supernatants other than *V. cholerae* with *E. coli* shown as a control. C) Comparison between cultures done in supernatants of different *V. cholerae* strains.

3.7 Conclusion

In this work a whole cell biosensor based on *V. cholerae* quorum sensing mechanism and CRISPRi technology for *V. cholerae* detection has been developed. As Cecchini et al. have pointed out in their recent review of *V. cholerae* detection methods, the gold standard for this detection is still the laborious culture method which is characterized by low sensitivity [177]. The more recent molecular methods (mainly *V. cholerae* DNA and toxin detection) which show the advantage of being more sensitive require the use of advanced equipment, limiting their use in affected areas. And most of the methods that are easy to use, such as LPS (Lipopolysaccharide) rapid diagnostic tests show either low specificity or sensitivity or even both.

Here, the sensor shows sensitivity to the presence of *V. cholerae* supernatant with tight control of expression of output GFP protein. In addition, the sensor design would be applicable for sensing other bacterial pathogen that has a unique sensing molecule like CAI-1. This could be achieved by replacing the quorum sensing proteins in the current design with other sensing proteins from an organism which produces similarly specific molecule. This work lays the foundation for future development of a highly sensitive, specific and easy-to-use whole cell biosensor for the detection of *V. cholerae*. In the current form this sensor requires only two specialized elements to be used – the genetically modified cells (potentially in lyophilized form for easier handling and longer shelf life) and basic fluorescence reading device. In the future, the GFP could be replaced with some other, easier to detect protein, or a protein that would produce a distinctive change in the medium (e.g. color or smell). Finally, the sensor can be developed further to produce anti-cholera infection molecules e.g. molecules targeting the cholera toxin or the *V. cholerae* cells themselves. This can turn the sensor into new *V. cholerae* treatment option.

Chapter 4 Adding killing capability to the *V. cholerae* biosensor

4.1 Introduction

Figure 4.1 Illustration of final engineered *E. coli* based *V. cholerae* killer biosensor working principle when the circuit is activated by CAI-1 produced by *V. cholerae*. CAI-1 by binding to CqsS results in its dephosphorylation followed by LuxU and LuxO dephosphorylation. Dephosphorylated LuxO is unable to activate expression of the repressor, which leads to unhindered expression of killer peptide. When killer peptide reaches the *V. cholerae* cells it makes it to die. Sensing module is shown in blue, inverting module is shown in red and actuating module is shown in orange.

The designed *E. coli* cells have been shown to be able to detect the *V. cholerae* supernatant. Before the next step of the design/construction process to achieve killing capability for the biosensor, a killing molecule which is able to efficiently kill *V. cholerae* cells had to be identified and characterized. Since the killing molecule will be produced inside the *E. coli* cell a suitable delivery system had to be prepared and characterized as well. Two possible systems can be considered – one where the killing system would be secreted out of the cells or one where the cell is forced to lyse thus releasing the payload. After these two components have been developed they had to be wired with the sensor circuit to create the final sensor-killer circuit by modifying the actuator module.

4.2 Search for killing protein able to kill *V. cholerae*

As a first step, it was required to determine what type of a killing peptide/protein could be used for *V. cholerae* eradication. As was mentioned in

Chapter 2, from many available options antimicrobial peptides/proteins were chosen for further investigation. Alternatives, like phages, were deemed too complex to be layered with the current system. Additionally, time which would be needed to design and test such a system was determined unsustainable. Antimicrobial peptide/protein on the other hand should have been relatively easy and fast to design and express. Two antimicrobials were considered for this tests – Microcin S and one of the Artilylins – Artilylin-085. Eventually Artilylin 085 was chosen. This was because Microcin was so far shown to be effective against quite a narrow spectrum of bacteria compared to Artilylin impressive broad spectrum efficacy.

First, it was required to determine whether expression of Artilylin-085 gene affects the growth of the host strain *E. coli*. This is important as the host system is designed to constitutively express and release the killing protein through lysis upon detection of *V. cholerae* since no secretion mechanism for Art-085 exists. Such a mechanism of delivery also has an added advantage of being relatively non-complex. Consequently, the killing protein must be benign to the host cell until the cell is lysed. For this, a plasmid expressing Art-085 under the regulation of the arabinose inducible pBAD promoter was constructed (Figure 4.2A). Upon induction with 0.2% arabinose, the *E. coli* host chassis expressing the Art-085 gene underwent no noticeable growth reduction compared to that of the control cells grown without arabinose induction (Figure 4.2B). This suggests that the Art-085 expressed intracellularly does not impact the growth of the *E. coli* host. This is most likely because it cannot reach the peptidoglycan layer from the inside of the cell. Based on this result, it was hypothesized that if a secretion protein (YebF) is tagged to Art-085 the resulting fusion protein can exert lysis activity on the host cell upon reaching the peptidoglycan layer and could be an effective lysis protein. To determine this, the YebF gene was fused to the N-terminal of Art-085 (YebF-Art-085) and it was placed under regulation of the pBAD promoter (Figure 4.2). As expected, when induced with 0.2% arabinose, the expression of YebF-Art-085 fusion protein

resulted in significant lysis of the host cells, presumably caused by YebF-Art-085 protein passing through the inner membrane thereby disrupting the peptidoglycan layer (Figure 4.2A).

Figure 4.2 Art-085 characterisation. A) Graphical depiction of the Art-085 testing circuit. The Artilysin-085 or its modified version of YebF-Art-085 are inducibly expressed by pBAD upon addition of arabinose. B) Plot showing changes in OD₆₀₀ of cultures of *E. coli* with plasmids expressing either Art-085 or YebF-Art-085 fusion. Cells expressing Art-085 grow at the same rate induced and uninduced. On the other hand, cells expressing yebF-Art-085 lyse rapidly upon induction.

Next, to examine the efficacy of Art-085 against the *V. cholerae* target strain, a plasmid carrying the Art-085 gene and Lysis E7 gene was constructed (Figure 4.3). As a control, a plasmid comprising only Lysis E7 gene but lacking the Art-085 gene was used. Upon induction with 0.2% arabinose, host cell carrying the Art-085 containing plasmid lyses due to Lysis E7 expression and releases the killing protein (Art-085) into the medium while the control cell also lyses, but without the killing protein release. To confirm the inhibitory activity of Art-085, both killer and control supernatants were filter sterilized and exposed to the *V. cholerae* test strain, respectively, and OD₆₀₀ was measured over time for 6 h. Cultures grown in the control supernatant lacking Art-085 showed normal growth while the cultures grown in the killing supernatant containing Art-085, showed high bactericidal activity against the *V. cholerae* test strain (Figure 4.3B). Dilutions of the collected supernatants to determine the

dose-response curves were also performed. It was found that $\geq 50\%$ dilution of the killer supernatants inhibited the growth of *V. cholerae*, while the control supernatant has no detrimental effect on the growth (Figure 4.3C,D). Overall, this shows that Art-085 can be stably expressed inside the *E. coli* host chassis and is effective at inducing *V. cholerae* cell death.

Figure 4.3 Art-085 killing efficacy characterization. A) Graphical depiction of the Art-085 killing efficacy testing circuit. Upon induction with arabinose both cells with and without Art-085 expression lyse due to E7 lysis peptide expression. B) Growth curve of *V. cholerae* cells grown in supernatant of *E. coli* cells carrying either Art-085 containing or control plasmid induced with 0.2% arabinose. As a comparison, the *V. cholerae* cells growth in fresh LB media is also shown. In the case of Art-085 containing supernatant induced with arabinose, the Art-085 proteins released upon lysis are able to inhibit the growth of *V. cholerae* cells rapidly upon exposure. In contrast, in the control supernatant induced with arabinose, the growth of *V. cholerae* cells is similar to the cells grown with fresh LB. C) Dose-response curve using the diluted supernatants of Art-085 expressing cultures induced with arabinose. From the data, it is evident that supernatant retains its killing properties up to 50% dilution. D) Same as C) but for control supernatant. IT shows that without the Art-085 expressed the *E. coli* cells growth is not inhibited.

4.3 Modification of the existing system with the lysing and killing devices

After characterising the reliability of YebF-Art-085 protein in lysing the *E. coli* host and the killing efficacy of Art-085 protein against *V. cholerae*, the next step was to integrate both the YebF-Art-085 and Art-085 genes into the existing *V. cholerae* sensor to create the complete the sense and eradicate system. First step of the integration was to replace GFP with YebF-Art-085 protein at the final point of cascade of the previous sensor design (in the actuator module). This would make lysis to be activated only with *V. cholerae* supernatant (CAI-1 present). One major difficulty in the process of replacing the constitutively expressed GFP from the sensor with YebF-Art-085 protein in the p15A origin plasmid was that the cells lysed during the transformation. Even with the co-transformation of the ColE1 origin plasmid expressing the gRNA to repress the constitutive promoter it was not possible to get the right transformants on the plate. As a result, it was concluded that the constitutive promoter used for driving GFP from the sensor could be too strong for YebF-Art-085 protein expression, meaning that the CRISPRi system could not prevent cell lysis due to the insufficient repression of the promoter (BBa_J23115) driving the YebF-Art-085 expression. Additionally, if it would be replaced with a weaker promoter the expression level of the lysis protein might be too weak, meaning that the cell will not lyse even upon *V. cholerae* detection. To circumvent this problem and to rapidly prototype the desired expression level, it was decided to modify the system (inverter module) so that the YebF-Art-085 protein will be expressed under an inducible promoter. Further, this will also enable to vary the expression level of YebF-Art-085 protein using the appropriate inducer to determine the optimal promoter strength. To do that the CRISPRi inverter's gRNA had to be redesigned to target new inducible promoter instead the previous constitutive promoter. The two considered promoters for this were the arabinose induced pBAD and ATc induced pTetR. One suitable gRNA was found for pBAD and two for pTetR. After the new gRNAs and promoters have been incorporated into the system the GFP could be finally replaced with YebF-Art-

Low cell density state

The diagram illustrates a genetic circuit designed for cell density-dependent gene expression. The circuit is shown in two states: Low cell density state and High cell density state.

Low cell density state: In this state, the CqsS receptor is active, leading to the production of CqsA, which inhibits the LuxU receptor. This results in the production of LuxO, which inhibits the tpQrr4 promoter, leading to the production of gRNA. The gRNA is then used by the Cas9 protein to target the YebF-Art-085 gene, leading to its deletion and the production of Art-085. The Art-085 protein is then used to produce the P15A protein.

High cell density state: In this state, the CqsS receptor is inactive, leading to the production of CqsA, which inhibits the LuxU receptor. This results in the production of LuxO, which inhibits the tpQrr4 promoter, leading to the production of gRNA. The gRNA is then used by the Cas9 protein to target the YebF-Art-085 gene, leading to its deletion and the production of Art-085. The Art-085 protein is then used to produce the P15A protein.

Legend:

- - RBS
- ⊥ - terminator
- pCon - constitutive promoter

Genetic Circuit Components:

- Top Plasmid (ColE1 origin):** Contains the CqsS gene (pCon), the LuxU gene (pCon), the p_{lacO-1} promoter, the LuxO gene (pCon), the tpQrr4 promoter, and the gRNA gene (pCon).
- Bottom Plasmid (P15A origin):** Contains the dCas9 gene (pCon), the pBAD promoter, the YebF-Art-085 gene (pBAD), the Art-085 gene (pCon), and the P15A origin.

Regulation:

- Arabinose:** Induces the expression of dCas9 from the pBAD promoter.
- gRNA:** Targets the YebF-Art-085 gene for deletion by Cas9.
- Art-085:** The product of the YebF-Art-085 gene, which is used to produce the P15A protein.

Next, three different strains of modified *E. coli* were prepared – one expressing the lysis protein under pBAD and two under pTetR (different gRNA).

The ones with pTetR promoter were found to be unusable, one of the strains were completely unable to stop the lysis (the strain lysed with the lowest tested ATc concentration) or unable to lyse (even adding very high ATc level did not lyse the cells, which would mean that the dCas9 binding was very strong). On the other hand, pBAD generated the expected results. Figure 4.5A shows the difference in OD₆₀₀ after 4h between the engineered *E. coli* cultures (with lysis device) grown in supernatants from cultures of two different bacteria: *E. coli*, non-activating (no CAI-1), as a control and *V. cholerae*, activating (CAI-1 present). The role of supernatants here is to simulate presence of the respective bacteria in the constructs growth environment. Following earlier reports [178, 179], cell lysis was defined as a visible negative change of OD₆₀₀ in time compared to the control culture. In line with this, cultures that are not growing i.e. showing no visible change of OD₆₀₀ in time are considered not lysing. From 0 to 0.001% of arabinose concentration, there is a visible inhibition of growth in cultures grown in *V. cholerae* supernatant, when compared to those cultured in the *E. coli* supernatant. However, there is no sign of lysis happening, which would mean that the pBAD is under-expressing the lysis protein. At arabinose concentration of 0.005% (Figure 4.5D) the desired effect has been achieved – culture with *E. coli* supernatant has its growth slowed down, but without trend suggesting lysis and the one cultured with *V. cholerae* supernatant shows evidence of lysis. Beyond the concentration of 0.005%, cultures grown both in *V. cholerae* and *E. coli* supernatants lyse, which would mean that the pBAD is able to express the YebF-Art-085 protein beyond the CRISPRi systems ability to suppress it. Nevertheless, this construct met the design criteria of having such an optimal arabinose concentration in which the activated construct (growing in *V. cholerae* supernatant) is lysing opposed to the control (*E. coli* supernatant).

Figure 4.5 Integrated sense, lyse and killing devices characterization in the engineered *E. coli*. A) Characterization of sense and lysis device (pSENSE + pKill2 plasmids). Bar graph showing differences in OD₆₀₀ of *E. coli* cultures carrying the pKill2 plasmid grown in either *E. coli* or *V. cholerae* supernatant under increasing arabinose concentrations. B,C) Characterization of sense, lysis and killing device B) Low starting inoculum concentration. C) High starting inoculum concentration (see page 110). Data were collected 4 h after induction with different arabinose concentrations. D) Growth curve of *E. coli* transformed with lysis device plasmids after induction with 0.005% of arabinose. E, F) Growth curve of *E. coli* transformed with pSENSE + pKill3 plasmids after induction with 0.001% of arabinose. E) Low starting inoculum concentration. F) High starting inoculum concentration. From this data, it is evident that *E. coli* cultures carrying either pKill2 or pKill3 plasmid when grown in *E. coli* supernatant (no CAI-1, ‘off’ state) grows normally. In contrast, the cultures grown in *V. cholerae* supernatant (CAI-1 present, ‘on’ state) starts lysis shortly after start of the culture thereby releasing the killing proteins.

After testing this circuit, the Art-085 killing protein expression was incorporated into the previous lysis device creating the killer device in the effect. Since it was already shown that the Art-085 can be safely expressed in *E. coli* under a constitutive promoter, it was decided to express it in this construct using an even stronger constitutive promoter with a strong RBS to ensure that high concentration of Art-085 will be produced and subsequently released upon cell lysis. Figure 4.4A,B show the full design of the system and how it behaves with and without CAI-1 in the extracellular environment.

Figure 4.5B shows the OD₆₀₀ difference after 4h between the engineered *E. coli* cultures (transformed with the killer device plasmids) grown in both *E. coli* (control) and *V. cholerae* (activating) supernatants. The system behaved similar to the one without the Art-085 incorporated, however there were some significant differences. First, general final OD₆₀₀ of the cultures were lower compared to the lysis-only cultures (when the killer protein expression was not present). Second, the desired characteristic of the culture was achieved in lower arabinose concentration, namely 0.001% (Figure 4.5E). Both effects could be due to the lysing cells releasing high amount of killing proteins (Art-085) which then accelerate the lysis rates of other cells by acting from outside the cells (positive feedback mechanism). To test this hypothesis, the cultures were repeated but with less dilution of the inoculum (see Materials and Methods section). This way higher starting inoculum at the point of addition of arabinose was achieved. If the hypothesis is right, then with higher OD₆₀₀ at the point of addition of arabinose, more Art-085 should be released with lysing cells and this positive feedback effect should be even more profound. The behaviour of such cultures with different arabinose concentrations are shown in Figure 4.5C. The striking difference is the constructs become more sensitive to *V. cholerae* supernatant in such conditions, where even without the addition of arabinose the culture shows obvious signs of lysis. With addition of 0.001% of arabinose the characteristic of the system is vastly improved, with cultures in *E. coli* supernatant growing to high OD₆₀₀ and ones in *V. cholerae* supernatant show fast lysis (Figure 4.5F). This could reinforce the view that it is very likely due to

Art-085 that improves the lysing capabilities and the effects seen are due to combined action of lysis and killing proteins.

4.4 Efficacy of the killer device against *V. cholerae*

With the full system constructed and characterized testing of its killing capabilities against *V. cholerae* could be initiated. The killing was tested with undiluted supernatants of the engineered *E. coli* cultures (carrying the full killer system plasmids) that were cultured separately in *E. coli* and *V. cholerae* supernatants and later induced with 0.001% arabinose. The supernatants were later inoculated with *V. cholerae* cells and moved to microplate for characterisation (Figure 4.6A). *V. cholerae* growing in fresh LB and the cultures with *E. coli* induced supernatant are showing similar growth rate. The cultures with the *V. cholerae* induced supernatant are on the other hand completely unable to grow, killing of cells is rapid and the results are similar to the one that was achieved in the previous tests. To check if this is not simply effect of supernatant itself (depletion of nutrients), a similar test with the construct which does not have Art-085 expressed has been also run (Figure 4.6B). In this test, only *V. cholerae* supernatant was used and the engineered *E. coli* cultures (carrying lysis device plasmids) were induced with high arabinose concentration (0.05%) to ensure complete lysis of the cells to simulate what happens in the killing construct (where full lysis is achieved in lower arabinose concentrations than in the lysis-only construct). The results show that even fully lysed, lysis-only cells supernatant are unable to impact the *V. cholerae* growth in any significant way, which would reinforce the notion that it is due to the assumed (because of strong promoter and RBS used for expression) high released concentration of Art-085 that is killing the *V. cholerae* cells.

Figure 4.6 Characterization of *V. cholerae* growth with the sense and eradicate system. **A)** Growth curve of *V. cholerae* cultures exposed to supernatant of *E. coli* cells carrying killer device plasmids, which were grown in either *E. coli* or *V. cholerae* supernatants induced with 0.001% arabinose. As a comparison, also shown is the *V. cholerae* cells growth in fresh LB media. Growth of *V. cholerae* cells in fresh LB and in *E. coli* supernatant induced cultures (carrying the killer device) progress similarly, whereas, the *V. cholerae* supernatant induced cultures (carrying the killer device) inhibits *V. cholerae* growth rapidly. This suggests that the engineered *E. coli* with sense, lyse and killing device in the genetic circuit can detect CAI-1 in the *V. cholerae* supernatant and responds by lysing the *E. coli* cells (by expressing YebF-Art-085) to release the killing proteins (Art-085) into the media. The released Art-085 in the supernatant inhibits the growth of *V. cholerae* cells rapidly. **B)** Growth curve of *V. cholerae* cultures exposed to supernatant of *E. coli* cells carrying the lysis device, which was grown earlier in *V. cholerae* supernatant induced with or without 0.05% arabinose. As a comparison, also shown is the *V. cholerae* cells growth in fresh LB media. Data shows that, no significant differences in growth rate of *V. cholerae* cells are noticeable with only the lysis device in the circuit. **C)** Time-kill curve analysis of *V. cholerae* cells grown in supernatant of *E. coli* cells carrying killer device plasmids, exposed previously to *E. coli* or *V. cholerae* supernatants induced with 0.001% arabinose. Cell viability was measured by CFU count after 14 h. Beyond the 60 min mark all *V. cholerae* cultures grown in *V. cholerae*

induced supernatant show levels of CFU below the methods lower detection limit of 100 CFU. D) Log reduction of CFU for the same cultures as in C). E) Agar overlay assay of *V. cholerae* cells after exposure to *E. coli* and *V. cholerae* induced supernatant of *E. coli* cells carrying the killer device. The photo shows the state of the plate after 4 h incubation. The labels are T1 and T2 for test 1 and 2 (two biological repeats) and Ec (*E. coli*) and Vc (*V. cholerae*) indicating supernatants. *V. cholerae* cells exposed to supernatant of the modified *E. coli* grown in *V. cholerae* supernatant displayed clear inhibition zones. This suggests that the system produces sufficient Art-085 to exhibit bactericidal activity.

To ensure that the *V. cholerae* cells are dead and not merely inhibited a time kill assay was performed, where the *V. cholerae* cultures exposed to the supernatants of the engineered *E. coli* carrying the final killer system grown in *E. coli* (control) or *V. cholerae* (activating) supernatants induced with 0.001% arabinose were plated on agar plates at different time points. The results in Figure 4.6C,D show the change in CFU of cultures grown in *E. coli* induced supernatant or *V. cholerae* induced supernatant. Cultures grown in *E. coli* induced supernatant shows expected steady growth in CFUs over time. On the other hand, *V. cholerae* induced supernatant cultures show drastic reduction in CFU starting at 30 min with plates at 60 min mark not showing any viable colonies grown. This is on par with results achieved for other bacteria tested against Art-085. To further visualize the inhibitory effects of the engineered *E. coli* on *V. cholerae*, an agar overlay assay was performed. The plate was divided into four parts and each quadrant had been added with three drops of supernatant of the engineered *E. coli* grown in either *V. cholerae* or *E. coli* supernatants and induced with 0.001% arabinose, before overlaying with soft agar mixed with *V. cholerae* cells. After 4 h of incubation there are evident plaques visible in the parts of the plate where the supernatant of the engineered *E. coli* induced with *V. cholerae* induced supernatant drops was added (Figure 4.6E). However, after 6 h the plaques were not visible anymore which could indicate limited thermal stability of Art-085 from the supernatant. Overall, the results suggest that the engineered *E. coli* carrying the final system, which includes CAI-1 sensing, YebF-Art-085 based lysing and Art-085 based killing device, can effectively inhibit the growth of *V. cholerae in vitro*.

4.5 Conclusion

In this work, first genetic circuit for *E. coli* that is capable of sensing *V. cholerae* and to which it responds with self-lysing to release the killing proteins that targets *V. cholerae* have been constructed and characterized. This was achieved by modifying the previously described *V. cholerae* sensor and layering it with lyse and kill circuit for *V. cholerae*. The mechanism of action is as follows: When *V. cholerae* specific QS signal (CAI-1) is present, the modified *E. coli* engineered with QS phosphorylation cascade detects CAI-1 resulting in the sensor proteins becoming dephosphatases and thereby LuxO is inactivated. Inactivated LuxO is unable to bind pQrr4 promoter leading to the down-regulation of gRNAs. Thus, CRISPRi activity is repressed leading to the expression of YebF-Art-085 protein inducing lysis of the host cell and thereby releasing the accumulated Art-085 (constitutively expressed). On the other hand, when CAI-1 is absent the phosphorylation cascade proteins engineered in the modified *E. coli* act as kinases and phosphorylated LuxO binds to pQrr4 up-regulating gRNA synthesis. Activated CRISPRi complex represses the YebF-Art-085 expression, resulting in no lytic activity thereby preventing the Art-085 release.

Figure 4.7 Artist impression of the working principle of the final system.

A step-by-step procedure to build this complex genetic circuit was followed [180]. First, it was identified that Art-085 can be expressed directly in the host cell without impacting its growth, which makes it suitable for bacterial delivery systems like the one presented in this chapter. Second, the killing potential of Art-085 against *V. cholerae* was characterized. According to the presented results Art-085 is able to eradicate the *V. cholerae* cells to the levels of lower than 100 CFU/ml within one hour, which is comparable with other known antibiotics, but possibly without the effect of resistance acquisition with time [89]. This also adds another bacteria species to the extensive list of organisms susceptible to the Art-085, which emphasizes its wide efficacy [87, 89, 90]. On the other hand, two possible drawbacks of Art-085 have to be underlined. The fact that the *V. cholerae* was able to overgrow the plaques visible on the agar plate assay after 6 h could presumably indicate Art-085 limited heat stability. Its very broad efficacy can also be seen as a drawback – it can possibly affect the commensal microbiome of humans which is highly undesirable. Third, the fusion of YebF secretion protein to the N-terminal of Art-085 was characterized. An interesting finding here is that addition of YebF to Art-085 potentially masks the Art-085 killing activity, as in the performed tests it was unable to inhibit growth of either *E. coli* or *V. cholerae* upon its release. However, this could be also due to low concentration of this lysis protein concentration in the supernatant after cell lysis has been completed. Possibly, this makes YebF-Art-085 an ideal lysis module as it induces lysis rapidly and can be used as a modular part to assemble novel synthetic circuits.

Once the individual killing and lysis devices were characterized the next step was to integrate them into the previous sensor circuit. The sensor device by was designed to be modular. The intent was to be able to replace GFP reporter in the actuator module with other genes/circuits such as the lysis and killing devices developed in this part of the work. The challenge here was that different genetic parts often require different expression levels to be effective. Because of incorporation of CRISPRi inverter it is easier for the system to be

redesigned for expression of other proteins as final points of the circuit requiring different expression levels. With CRISPRi, one can simply find, or design, a suitable gRNA-promoter pair for the task. The short length of parts to be changed (tens of base pairs) for this pair replacement is also of big advantage when DNA manipulation is considered. In the case of this study, it was achieved by replacing the constitutively expressing signal protein (GFP) from the previous sensor with the arabinose-inducible pBAD promoter to drive the lysis protein production. In order for this to work, the previous sensor was modified to express anti-pBAD gRNA under the pQrr4 promoter. Coupling of CRISPRi with inducible pBAD promoter enabled a very rapid prototyping of the system. Alternatively, creating a library of different constitutive promoters to find one that would express the lysis protein on high enough level to lyse the cell and be readily repressed by the dCas9 complex at the same time would be labour intensive and time consuming. By this approach of using an inducible promoter, it was possible to test different expression levels at the same time which cut down testing time considerably.

Similar system, but for *Pseudomonas aeruginosa* has been constructed and discussed in this worked earlier [106]. However, most possibly this is the first time a synthetic *V. cholerae* sense and kill gene circuit has been developed for commensal *E. coli*. Compared to that previous work this sensor uses much more elaborate sensing system (based on two-component system) and introduces a CRISPRi based genetic inverter. In terms of killing it introduces use of broad-spectrum, efficient antimicrobial protein which can be repurposed for eradication of other bacteria. Also, the lysis part of the circuit has been improved by introduction of new lysis protein with faster cell lysis.

The genetic circuit presented in this work may be seen as a first stage of a process aiming at developing a new therapy option for *V. cholerae*. But before this can be done, there are a few challenges to overcome first – the two-plasmid system with two separate selection antibiotics are not a sustainable option for

prolonged clinical studies. The answer would be integrating the whole circuit with the bacterial genome, however this may prove hard due to the number of elements involved. Next, it would be beneficial to use *E. coli* Nissle 1917 strain instead of the wild-type MG1655 strain due to Nissle's proven track of being benign to humans [181]. Also, Art-085 impact on the intestinal microbiome would have to be studied. Importantly, for future implementations studying the *in vivo* efficacy of the engineered *E. coli* in direct disease relevant animal models is necessary to elucidate its effect as a combined prophylactic and therapeutic against cholera. Nevertheless, presented data shows that the concept is viable and with further development this could be used to save thousands of human lives in the near future.

Chapter 5 Summary and future works

5.1 Summary

In this thesis, I have presented studies performed towards designing and creating a genetic circuit for *E. coli* to enable it to sense and kill *V. cholerae*. This work shows how a problem of constructing an “inverted biosensor” can be approached with engineering mindset. It shows how the development is to be divided into modules that can be characterized apart from each other and then integrated into higher level device of more elaborate function. Through this process of design a novel biosensor for *V. cholerae* detection and eradication has been created in *E. coli*. This work elucidated how the parts of the two-component quorum sensing system perform and how these parts can be layered with CRISPRi inverter/amplifier with an aid of computer model. This enabled construction of first known whole-cell based *E. coli* biosensor for *V. cholerae* detection. Additionally, it shows how the first known *V. cholerae* killing device has been characterized, constructed and integrated with the aforementioned biosensor.

The work has been divided into a set of steps, which allowed systematic approach to the systems design and characterization. Later, the most basic parts were organized into three modules (sensor, inverter and actuator) which were finally layered to create the final device.

Before the *V. cholerae* killing capability could have been developed, a robust *V. cholerae* sensor had to be created. The first step to achieve this was characterization of pQrr4, a promoter which would become an interface between the sensor and inverter modules. This was this promoter’s first characterization in *E. coli*, which showed that it does not show very high specificity towards phosphorylated LuxO. Since it was crucial for it to be so; the promoter had been modified to increase its specificity dramatically.

The next step in creation of the sensor was the design and characterization of the inverter module. Several designs were considered for the inverter

module. First, natural extension of *V. cholerae* quorum sensing system in form of HapR and pAphA was characterized. However, its properties were found to be lacking – such an inverter did not show high enough repression to be able to repress the signal protein expression. Next, a library of other TetR family repressors were considered, but it was decided too time consuming to prepare the required library of repressors, especially that new effective alternative has been found. This alternative was the CRISPRi technology which allowed to prepare a set of different promoters of different strengths with effective repression.

With inverter module designed and characterized the works on sensor module could begin. A set of similar constructs was prepared where the three quorum sensing related proteins from *V. cholerae* were expressed on different levels. Data from characterization of these constructs were fed to a computer model for analysis. This analysis showed which components expression levels are crucial for the sensor module and enabled its optimized design.

With sensor and inverter modules characterized their layering with the actuator module (in sensor device case it was GFP with its RBS) could begin. Since the strength of the pQrr4 was already known, the right promoter-gRNA pair of all the tested ones could be chosen. Using data from the model optimal configuration of quorum sensing proteins was derived for the chosen inverter. Finally, GFP was introduced as the endpoint of the cascade. Finally, this construct was characterized and shown to be reacting to *V. cholerae* supernatant by producing GFP output.

Next, with the sensor device working, the *V. cholerae* killing mechanism could be developed. From the possible strategies one that relies on host cell lysis and killing peptide release has been chosen. For the killing, Artilysin-085, an antimicrobial peptide has been chosen. After proving its ability to kill *V. cholerae* cells, it could become, together with YebF-Art-085 lysis peptide, the replacement for GFP as the actuator module. After integration of this new

actuator module with the modified inverter module the whole device was characterized – first its lysing ability upon *V. cholerae* supernatant detection and later its killing capability against *V. cholerae* cells.

5.2 Future works

This work has provided future research with a robust platform for development of potential commercial *V. cholerae* detection and therapy option. But before this, a few studies would have to be done.

First, the molecular components of the systems can be further optimized to increase system's sensitivity, selectivity and sensing/killing capability. This could be achieved by modifying CqsS, levels of sensor protein expression or further optimizing the inverter module.

Second, currently the achievements of this Thesis can be only shown to be working *in vitro*, both the sensor and killing devices. This means that the natural extension of the works would be *in vivo* and other animal model feasibility studies.

Looking even further into the future one can image the system as a development platform for other microbial systems with sense and kill capabilities. The universal characteristics of the inverter module (since CRISPRi can be easily adapted for different promoters) and the Artilysin-175 (it was shown before to be effective against a range of different bacteria) should enable easy redesign of the parts of the system to adapt it to use quorum sensing of other bacteria, which are meant to be detected and/or killed.

5.2.1 Optimization of molecular components of the developed genetic devices.

First element that could be optimized molecularly is the sensor protein CqsS. According to Ng et al. mutating CqsS by changing the 31st amino acid from proline to alanine (P31A) makes the CqsS to turn into dephosphatase in concentrations of CAI-1 lower by two orders of magnitude than wild type, which would make the devices to activate at lower *V. cholerae* density in the

environment which would greatly improve the sensitivity. The main difficulty with this mutation may be the fact that expression level of other components of the system will need to be readjusted to accommodate the change in the sensitivity.

Next, the CRISPRi inverter module could be optimized. From the multitude of available constitutive promoters for the sensor device, only four have been tested. From this only one has been chosen to be used in the designed device, which had the most promising characteristic. There is however a very high possibility that other, more suitable promoters can be found. They could be stronger than the one chosen with the same or better repression characteristic. The same logic applies for the sense and kill device, the pBAD promoter can be replaced now with a constitutive one of strength corresponding to the strength of pBAD induced with used arabinose levels. The main obstacle to overcome here would be the fact that without a very precise *in silico* model finding the right promoters will involve creating big libraries of constructs which would require huge amount of time and effort to construct and test.

In case of the sensor device some other reporting mechanism could be considered instead of GFP. The main disadvantage for use of GFP is the need of using of specialized equipment for making the appropriate reading. This could pose a problem when the sensor would be used in the field. To mitigate this some kind of enzyme could be incorporated instead of or alongside GFP. For instance, an enzyme responsible for producing some chemical of very distinctive smell could be used. However, this may require ensuring its high expression rate and possibly supplementation of the growth media with a suitable substrate to be turned into the detected molecule.

Finally, the killing device could be improved by changing the killing molecule so that it can be secreted out instead of relying on lysis of the cell for delivery. Without the cells needed to be lysed the system should be much more

persistent – with lysed cells the potential therapy would have to be repeated after each *V. cholerae* infection and without a definite sign that another dose is needed one can become vulnerable to infection without knowing. This secretion type of delivery could be achieved by creating an Art-085 fusion with a suitable secretion tag or by changing Art-085 to some other bacteriocin with natural secretion system. However, with such a system an immunity will have to be introduced to the cells since once the killing molecule is secreted it will be able to attack the host cell.

5.2.2 *In vivo* studies

After showing the circuit to be working *in vitro* the natural next thing after molecular optimization of the system would be moving into *in vivo* studies in mice. However, before this can be done, besides the optimization steps introduced in the previous subchapter some other points would need attention:

- a) First and foremost, the fact that the current system is based on two plasmids having two distinct, antibiotic selection systems will be unsuitable for prolonged *in vivo* testing. One option would be changing the antibiotic systems into auxotrophic selection systems and then supplying suitable nutrients with the medium. Even more desirable option would be integrating the whole circuit with the bacterial genome to ensure its high stability. However, due to systems size this will need several steps of genome integration and high number of tests to ensure that systems performance is not degraded
- b) The strain of *E. coli* used in the presented studies is the MG1655 wild type. It would be very advantageous to replace it with Nissle 1917 strain [182]. Nissle 1917 is a probiotic strain of *E. coli* which is marketed as Mutaflor by Australian company Natural Therapy Imports. Besides being proven to be effective bowel disease medication [183], it was also showed that Nissle 1917 can be used

as induced deliverer of medicinal compounds directly to the human gut [184]. Induction of Nissle was achieved by oral administration of inducing compounds to mice test subjects previously showing genetically modified Nissle presence in intestines [184]. This change would probably force changes in the promoters or RBS for some components of the system due to generally different expression levels which happen from one strain to another.

- c) Artilysin – 085 needs to be tested against a host of other bacteria living within the human intestines. It would be highly disadvantageous if as the therapeutic is released upon *V. cholerae* detection other benign/symbiotic bacteria would be killed. This could leave the patient vulnerable to other opportunistic infections due to lack of the natural defense provided by this natural microbiota. In case this would be found to be a case other bactericidal system would have to be found.
- d) Similarly, possible lack of specificity of the sensor has to be investigated, so that it is ensured it won't be triggered by other bacteria that are part of the normal human microbiota
- e) Finally, the matter of bacteria localization will have to be raised. *E. coli* is normally colonizing human large intestine and colon, whereas *V. cholerae* is known to inhabit the small intestine. Another problem is the fact that *V. cholerae* is normally embedded in the mucus of the intestine which could potentially limit the diffusion of the killing molecule towards them. This could be solved by moving the whole system to other bacteria with similar ecological characteristics. In fact, a good candidate would be another *V. cholerae* strain that is shown not to be able to produce the cholera toxin or to acquire this ability via transfection. However, this would require redesign of the circuit for the use in different organism which would be a major endeavor.

With these points considered the system could move into another step towards becoming a new therapy option for *V. cholerae* infection.

5.2.3 Repurposing the system for detection of other bacteria

Artilysin - 085 is a broad spectrum bactericidal protein. This can be a disadvantage as mentioned in the previous subchapter, but at the same time it becomes one of the strengths of the system. Since it was proven to work against a range of human pathogens the killing device could be easily repurposed to be working against different bacteria genus. The main change required to do this will be changing the parts of the sensor module to proteins comprising quorum sensing system of another bacterium.

Since CqsS and CAI-1 are conveying very specific to *V. cholerae* signal the first in a series of experiments could be changing it to LuxPQ of *V. cholerae*. This system is known to detect AI-2 much more widely used molecule for quorum sensing, but at the same time uses LuxU and LuxO which would minimize part of the system to be redesigned. This should enable the device to detect other *Vibrios* and other closely related species using the same type of AI-2 molecule (borate based). Similarly, other *V. cholerae* sensors and *V. harveyi* sensors using LuxU for phosphotransfer could be investigated for specificity against other bacteria.

Many Gram-positive bacteria are also known to use similar two component system for quorum sensing, with the difference being that the signaling molecule in this case is a short peptide. This would require more extensive modification, down to the promoter for the gRNA. Not all those systems also require inversion of the signal along the way. Additionally, Atrilysin-175 shows only moderate efficacy against the Gram positives, which would may require its replacement as well.

Chapter 6 Methodology

6.1 Plasmids - design and construction

All the plasmids used in the study were first designed *in silico* and then constructed using standard assembly (following Sambrook manuals [185]) or isothermal assembly (Gibson assembly [186]) protocols . DNA fragments used for construction were either synthesized, PCRed in-house or bought (in case of backbone plasmids). All transformations were done into *E. coli* cells.

6.1.1 In silico design

All the plasmid sequences for the pQrr4 and HapR inverter study were developed using the NTI software (Thermo Fisher, Waltham, MA, USA). All the subsequent designs were done in Benchling web-based designer (Benchling, San Francisco, CA, USA). The sequences of proteins, RBS, promoters and terminators used in the study came either from NCBI Genbank or iGEM databases [171, 172, 187]. After acquiring the sequences, they were assembled *in silico* using the design wizards for respective methods in the aforementioned software. All the created plasmids were stored as separate Genbank compliant entries in the databases of the respective software.

6.1.2 DNA synthesis

The sensor plasmids for the combinatorial studies were synthesized as whole, ready to use plasmids by GenScript (Piscataway, NJ, USA). The DNA sequences for CqsS, LuxU and LuxO were later PCRed from these sensor plasmids. If there was a need for a shorter stretch of sequence that would not justify ready to use synthesis a gBlock was used instead, synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). The same company supplied all the PCR primers used in the study.

6.1.3 Bgl brick standard assembly

The BglBrick is a BioBrick derivative standard created and described by Lee et al. [110, 188]. The BioBrick standard aims at easing the process of assembly by providing a set of four fixed restriction sites which are shared between the

backbones and genetic parts. This way a standardized protocol can be used each time for every assembly making it faster, easier and cheaper. The main modification of Bgl Brick over BioBrick is the possibility of creating fusion proteins with ease by choosing restriction sites that create a scar that ensures non-disrupted transcription over it.

6.1.4 Gibson assembly

Isothermal assembly or Gibson assembly is an DNA assembly method which uses a mix of enzymes (exonuclease, polymerase and ligase) for easy and efficient joining of DNA fragments. Isothermal assembly used in this study mostly followed the manufacturers manual, with extension of assembly time for long fragments being the most common modification.

6.1.5 Plasmid backbones

The Bgl Brick founders provide a series of backbone plasmids to be used with the standard. They can have different site of origin, antibiotic resistance and promoter used for expressing the proteins (all inducible). Of all the plasmids provided this study used primarily four of them as backbones and source for standard sequences for sensor plasmids – pBbE8k, pBbE8a, pBbE6c and pBbE6k. For the CRISPR matrix study two backbones provided by Qi et al. were used – pdcas9 and pgrna-bacteria [151, 154]. All the backbone plasmids were provided by Addgene (Cambridge, Massachusetts, USA).

6.1.6 DNA sequencing

All DNA sequencing services (only of Sanger type) were supplied by 1st Base Company (Singapore). All sequencings were done using either standard primers provided by the company or ones created by the needs of the study. After sequencing the acquired sequences were aligned using Vector NTI or Benchling align tool.

6.1.7 Maps of plasmids used in this study

Only the maps for plasmids used in the experiments are shown, without the ones used as intermediate construction steps.

a) Plasmids used for actuator module study

A1 – A4 plasmid – combined map of plasmids for LuxO and LuxOt characterization with full and truncated Qrr4 promoter.

b) Plasmids used for the HapR based inverter study

B1 plasmid – for pAphA characterization

B2 -B5 plasmids – set of plasmids to test the impact of four Qrrs on HapR expression

B6 plasmid – for demultiplexer tests

c) Plasmids used in the study of CRISPRi inverter

C1-C4 plasmid – example of plasmid harboring dCas9 and GFP for CRISPRi system testing. Each of the four plasmids had different Anderson constitutive promoter used for GFP expression.

C5-C12 plasmid - example of plasmid harboring gRNA for CRISPRi system testing. Each of the eight plasmids had different gRNA sequence.

C13 plasmid – plasmid used for constitutive dCas9 expression study. Also, it was used as receiver plasmid for double plasmid full integrated system (with plasmid D1).

d) Combinatorial sensor plasmids for *in silico* model

D1 plasmid – plasmid used for combinatorial sensor study as sensor plasmid.

D2 plasmid – plasmid used as a receiver plasmid in combinatorial sensor study.

e) The sensor device plasmid

E1 plasmid – the inverted sensor plasmid for sensor device full integrated system (Also, please see plasmid C13).

f) Plasmids used for Art-085 and YebF-Art-085 study

F1 plasmid – plasmid inducibly expressing Art-085

F2 plasmid – plasmid inducibly expressing YebF-Art-085

F3 plasmid – control plasmid inducibly expressing E7 lysis peptide

F3 plasmid – control plasmid inducibly expressing E7 lysis peptide and constitutively expressing Art-085

g) Plasmids used for the killing device

G1 plasmid - actuator plasmid used with G3 to form the lysis-only device

h) Plasmids used to create the *in silico* model for killing device

This study used two species of Gram negative bacteria, namely *Escherichia coli* and *Vibrio cholerae*. All the transformations have been done in the TOP10 or Beta 10 strains of *E. coli*. All the characterizations were done in MG1655 (K-12) strain of *E. coli*. For *Vibrio cholerae* three strains have been used – El Tor clinical isolate, A1522 GFP producing strain and Δ HapR strain which is unable to turn on and off its quorum sensing mechanism.

The following subchapters present the protocols used for microplate readings for the experiments. They are given in order in which they are used in the main text. All the readings were done in Biotek Synergy HT microplate reader. The

reader settings used were as follows: Wavelength – Excitation: 485nm, 488nm
Emission: 528nm, 532nm for GFP and RFP respectively; Optics position: top;
Sensitivity: 35; Vertical Offset: 1mm; Path Length Correction: Off.

6.3.1 Protocol for actuator module experiments

Overnight liquid LB culture (5mL in 50mL tube, 37 °C) of *E. coli* with tested plasmid supplemented with suitable antibiotic was diluted 100 times in supplemented LB and this diluted culture (1mL per measured inducer concentration in 50mL tube, 37 °C) was left in an incubator for between 1.5 and 2.5 hours. Culture after reaching OD₆₀₀ of between 0.1 – 0.2 was moved to 96-well, transparent, flat-bottom microplate and distributed in triplicates at 300 µL to each well. Arabinose was added directly into the wells. The constructs have been tested in microplate with arabinose concentrations of: 0, 0.000005, 0.00001, 0.0001, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.075 and 0.125%. Microplate reading was continued with vigorous shaking for 6 hours, with OD₆₀₀ and GFP fluorescence reading taken every 10 minutes. Relevant control and blank measurements were taken in each microplate session.

6.3.2 Protocol for HapR based inverter study

All the overnight liquid LB culture (5mL in 50mL tube, 37 °C) of *E. coli* supplemented with suitable antibiotic with tested plasmid was diluted 100 times in supplemented M9 and this diluted culture (1mL per measured inducer concentration in 50mL tube, 37 °C) was left in an incubator for around 2.5 hours. Culture after reaching OD₆₀₀ of 0.2 was moved to 96-well, transparent, flat-bottom microplate and distributed in triplicates at 300 µL to each well. If inducer was needed it was added to the well before the culture. Arabinose concentrations examined in all measurements were: 0, 0.000005, 0.00001, 0.0001, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.075 and 0.125%. Microplate reading was continued with vigorous shaking for 6 hours, with OD₆₀₀ and RFP fluorescence reading taken every 10 minutes. Relevant control and blank measurements were taken in each microplate session.

6.3.3 Protocol for CRISPRi inverter study

Overnight liquid LB culture (5mL in 50mL tube, 37 °C) of *E. coli* with tested plasmid supplemented with suitable antibiotic was diluted 100 times in supplemented LB and this diluted culture (1mL per measured inducer concentration in 50mL tube, 37 °C) was left in an incubator for between 1.5 and 2.5 hours. Culture after reaching OD600 of between 0.1 – 0.2 was moved to 96-well, transparent, flat-bottom microplate and distributed in triplicates at 300 µL to each well. Each CRISPRi construct (with one of the pdCas9 plasmids and of the gRNA plasmids) has been tested in 2D matrix in microplate with ATc concentrations: 0, 0.1 nM, 0.25 nM and 0.5 nM and IPTG concentrations 0, 3 µM, 6 µM, 12 µM, 25 µM, 50 µM, 100 µM and 500 µM. Microplate reading was continued with vigorous shaking for 6 hours, with OD600 and GFP fluorescence reading taken every 10 minutes. Relevant control and blank measurements were taken in each microplate session.

6.3.4 Protocol for sensor device study

Overnight liquid LB culture (5mL in 50mL tube, 37 °C) of *E. coli* with tested plasmid supplemented with suitable antibiotics were diluted 100 times in supplemented LB with appropriate supernatants and this diluted culture (1mL per measured supernatant/inducer concentration in 50mL tube, 37 °C) was left in an incubator for between 1.5 and 2.5 hours. Culture after reaching OD600 of between 0.1 – 0.2 was moved to 96-well, transparent, flat-bottom microplate and distributed in triplicates at 300 µL to each well. Constructs have been tested with 0, 25, 50, 100, 200, 300 or 500 uM IPTG and 0, 5%, 30%, 50% or 90% of supernatants of *V. cholerae* A1522, and *E. coli* K-12. IPTG was added to the culture tubes before the outgrowth phase. Supernatants have been prepared by culturing overnight respective bacteria in LB and then removing the bacteria by centrifugation and filtering using 0.22 µm syringe filters. For cultures containing 90% of either supernatant the media has been topped up with two times concentrated LB. Microplate reading was continued with vigorous shaking for 6 hours, with OD600 and GFP fluorescence reading taken every 10 minutes.

Relevant control and blank measurements were taken in each microplate session.

6.3.5 Protocol for Art-085 and YebF-Art-085 protein study

Both the overnight grown strains (5mL in 50mL tube, 37 °C) were diluted 100 times in fresh LB supplemented with gentamicin and moved to a shaking incubator for 3 h outgrowth to reach an OD₆₀₀ of 0.7-1. The cultures (1mL per measured supernatant concentration in 50mL tube, 37 °C) were induced with 0.2% arabinose and were allowed to grow for another 4 h in order to complete the lysis process. The supernatant was collected after centrifugation and filtration using 0.22 µm Millipore membrane filter unit. Parallely, pre-grown *V. cholerae* test strain was diluted to 0.5 OD₆₀₀ using fresh LB media supplemented with gentamicin. In order to determine the dose-response of both the killer and control supernatants, dilutions ranging from 75%, 50%, 25%, 12% and 6% with fresh LB medium were performed. The collected and diluted supernatants were then mixed with the prepared *V. cholerae* target cells in 1:9 ratio (180 µl of respective supernatants with 20 µl of *V. cholerae* cultures) and aliquoted in a 96-well plate. Subsequently, the cultures were moved to the microplate and tested in the reader for another 6 h.

6.3.6 Protocol for killing device study

Characterization assay of *E. coli* cells harboring either lysis-inducing or final system was carried as follows – overnight cultures in LB (5mL in 50mL tube, 37 °C) of *V. cholerae* and *E. coli* were sub cultured by diluting 100 times and was transferred into the shaking incubator for 4 h outgrowth (10mL in 50mL tube, 37 °C). After this the supernatant from the cultures was obtained by centrifugation and filter sterilized using 0.22 µm Millipore membrane filter. Subsequently, overnight cultures of tested constructs were diluted 100 or 25 (for high starting OD₆₀₀ tests) times in the respective supernatants (10mL in 50mL tube, 37 °C). Such cultures were first moved to the shaking incubator for

2 h outgrowth and then were supplemented with inducers and moved to the microplate and tested in the reader for another 6 h.

The killing assays were carried out in the following way: overnight grown cultures (5mL in 50mL tube, 37 °C) of *V. cholerae* and *E. coli* were recultured by diluting 100 times in fresh LB and were transferred into the shaking incubator for 4 h outgrowth (10mL in 50mL tube, 37 °C). After this the supernatants from the respective cultures were collected by centrifugation and filter sterilized with 0.22 µm Millipore membrane filter. Next, overnight cultures of the engineered *E. coli* carrying either lysis-inducing or the final system were diluted 25 times in the respective supernatants with appropriate antibiotics (ampicillin and chloramphenicol). The cultures were first moved to the shaking incubator for 2 h outgrowth and then were supplemented with inducers and kept in the shaking incubator for another 3 h to complete the lysis process. After 3 h, the supernatants from the respective cultures were collected by centrifugation followed by filtration with 0.22 µm Millipore membrane filter. Parallely, overnight grown *V. cholerae* target cells were diluted to an OD₆₀₀ of 0.5 with fresh 2xLB. The collected supernatants were then mixed with the prepared *V. cholerae* (*Vc-hapR*-RFP) target cells in 1:9 ratio (180 µl of respective supernatants with 20 µl of *V. cholerae* cultures) and aliquoted in a 96-well plate. Subsequently, the cultures were moved to the microplate and incubated in the reader for another 6h.

The time kill assay was done by preparing serial dilutions of *V. cholerae* cultures exposed to the supernatants of the engineered *E. coli* carrying the final system grown in *E. coli* (control) or *V. cholerae* (activating) supernatant induced with 0.001% arabinose that were run in tubes in a shaking incubator in parallel with microplate assay. The dilutions were made for: inoculum, 0, 30, 60, 90, 120, 180, 240, 300 and 360 min time points and with dilutions ratios from 10² to 10⁹ times. That set the lower detection limit to 100 CFU and higher detection limit

to 10^9 CFU. The dilutions were plated on LB plates without antibiotics, incubated overnight in 37°C for 14h after which colony count was performed.

The agar overlay assay was done by preparing an LB agar plate, dividing the plate into four equal quadrants, with each of them having three 20 μ L droplets of supernatant (either from *E. coli* or *V. cholerae* supernatant induced cultures of the engineered *E. coli* carrying the final system with 0.001% arabinose added. After the droplets have dried up, overnight cultures of *V. cholerae* target cells were homogeneously mixed with the soft LB agar solution (10 g/l tryptone, 5 g/l yeast extract, 10 g/l sodium chloride and 7 g/l agar) and gently poured on to the agar plate. The agar plate with the soft agar overlaid was kept in the incubator at 37°C and photograph was taken under a white light source after 4 h of incubation.

6.4 Data Analysis

All the fluorescence and absorbance data was analyzed as follows – from each fluorescence and absorption value an averaged blank value was subtracted. Next, for each sample in triplicate, for each time point, the fluorescence value was divided by the respective absorbance value. Next, these values were averaged for each triplicate and standard deviation for each sample was calculated. If normalization was required it was done by dividing the RFP/OD₆₀₀ values of each sample by the value for the sample with highest value. Since this method divides one average by another a standard deviation value is not possible to calculate.

6.5 Software

The model was developed and analyzed using Matlab with Simulink (The MathWorks, Inc., Natick, Massachusetts, USA). Graphs were made using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, California, USA). Data analysis was done in Microsoft Excel (Microsoft, Redmont, Washington, USA).

6.6 Consumables

NEBuilder master mix for Gibson assembly has been supplied by New England Biolabs (Ipswich, Massachusetts, USA). For PCR either iProof PCR kit supplied by Bio-Rad (Hercules, California, USA) or Q5 PCR kit supplied by New England Biolabs (Ipswich, Massachusetts, USA) were used. Purification kits (PCR, Gel, Miniprep) were supplied by Axygen (Tewksbury, Massachusetts, USA) and Qiagen (Hilden, Germany). DNA concentrations were checked using Nanodrop ND-1000 spectrophotometer. Commercial M9 and LB broth and agar supplied by BD (Franklin Lakes, New Jersey, USA) were used in all the studies. Supplemented M9 (M9 salts, 1 mM thiamine hydrochloride, 0.8% glycerol, 0.2% casamino acids, 0.1 M MgSO₄, 0.5 M CaCl₂) or LB was used as the medium for the characterization.

Chapter 7 References

1. Meehan, C.D.M., Howard, *Cholera, Historical*. The Encyclopedia of Microbiology, 2000. I.
2. Azman, A.S., et al., *The incubation period of cholera: a systematic review*. J Infect, 2013. **66**(5): p. 432-8.
3. Hempel, S., *John Snow*. The Lancet, 2013. **381**(9874): p. 1269-1270.
4. Howard-Jones, N., *Robert Koch and the cholera vibrio: a centenary*. Vol. 288. 1984. 379-381.
5. Who, *Weekly epidemiological record*. 2014. **31**: p. 345-356.
6. Harris, J.B., et al., *Cholera*. The Lancet, 2012. **379**(9835): p. 2466-2476.
7. Kothary, M.H. and U.S. Babu, *INFECTIVE DOSE OF FOODBORNE PATHOGENS IN VOLUNTEERS: A REVIEW*. Journal of Food Safety, 2001. **21**(1): p. 49-68.
8. Matson, J.S., J.H. Withey, and V.J. DiRita, *Regulatory networks controlling Vibrio cholerae virulence gene expression*. Infection and Immunity, 2007. **75**(12): p. 5542-5549.
9. Chun, J., et al., *Comparative genomics reveals mechanism for short-term and long-term clonal transitions in pandemic Vibrio cholerae*. Proc Natl Acad Sci U S A, 2009. **106**(36): p. 15442-7.
10. WHO, *The treatment of diarrhoea A manual for physicians and other senior health workers*. 2005: p. 44.
11. Yam, W.C., M.L. Lung, and M.H. Ng, *Evaluation and optimization of a latex agglutination assay for detection of cholera toxin and Escherichia coli heat-labile toxin*. Journal of Clinical Microbiology, 1992. **30**(9): p. 2518-2520.
12. Colwell, R.R., et al., *Development and evaluation of a rapid, simple, sensitive, monoclonal antibody-based co-agglutination test for direct detection of Vibrio cholerae O1*. FEMS Microbiol Lett, 1992. **76**(3): p. 215-9.

13. Harris, J.R., et al., *Field evaluation of crystal VC Rapid Dipstick test for cholera during a cholera outbreak in Guinea-Bissau*. Trop Med Int Health, 2009. **14**(9): p. 1117-21.
14. Marquess, D., *Recent Advances in Discovery and Development of Medicines for the Treatment of Secretory Diarrhea in the Developing World*, in *Third World Diseases*. 2011, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 277-300.
15. Eisenberg, M.C., et al., *Examining rainfall and cholera dynamics in Haiti using statistical and dynamic modeling approaches*. Epidemics, 2013. **5**(4): p. 197-207.
16. Pastor, M., J.L. Pedraz, and A. Esquisabel, *The state-of-the-art of approved and under-development cholera vaccines*. Vaccine, 2013. **31**(38): p. 4069-78.
17. Focareta, A., et al., *A recombinant probiotic for treatment and prevention of cholera*. Gastroenterology, 2006. **130**(6): p. 1688-95.
18. Duan, F. and J.C. March, *Engineered bacterial communication prevents Vibrio cholerae virulence in an infant mouse model*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(25): p. 11260-11264.
19. Rodríguez-Rojas, A., et al., *Antibiotics and antibiotic resistance: a bitter fight against evolution*. International Journal of Medical Microbiology, 2013. **303**(6-7): p. 293-297.
20. Mrozek-Budzyn, D., R. Majewska, and A. Kieltyka, *Early exposure to thimerosal-containing vaccines and children's cognitive development. A 9-year prospective birth cohort study in Poland*. Eur J Pediatr, 2014.
21. Tatem, A.J., D.J. Rogers, and S.I. Hay, *Global Transport Networks and Infectious Disease Spread*, in *Advances in Parasitology*, A.G. Simon I. Hay and J.R. David, Editors. 2006, Academic Press. p. 293-343.
22. Milton, D.L., *Quorum sensing in vibrios: complexity for diversification*. Int J Med Microbiol, 2006. **296**(2-3): p. 61-71.
23. Zhu, J., et al., *Quorum-sensing regulators control virulence gene expression in Vibrio cholerae*. Proc Natl Acad Sci U S A, 2002. **99**(5): p. 3129-34.
24. Jung, S.A., C.A. Chapman, and W.L. Ng, *Quadruple quorum-sensing inputs control Vibrio cholerae virulence and maintain system robustness*. PLoS Pathog, 2015. **11**(4): p. e1004837.
25. Deane, C., *Synthetic biology: Cholera detectors*. Nat Chem Biol, 2016. **12**(11): p. 889-889.
26. Zhang, Q.Q., et al., *Inhibition of biofilm formation of Pseudomonas aeruginosa by an acylated homoserine lactones-containing culture extract*. LWT - Food Science and Technology, 2014. **57**(1): p. 230-235.
27. Ng, W.L. and B.L. Bassler, *Bacterial quorum-sensing network architectures*. Annu Rev Genet, 2009. **43**: p. 197-222.
28. Diggle, S.P. and P. Williams, *Quorum Sensing*, in *Brenner's Encyclopedia of Genetics (Second Edition)*, S.M. Hughes, Editor. 2013, Academic Press: San Diego. p. 25-27.
29. Bassler, B.L., *Small Talk: Cell-to-Cell Communication in Bacteria*. Cell, 2002. **109**(4): p. 421-424.
30. Bassler, B.L. and R. Losick, *Bacterially speaking*. Cell, 2006. **125**(2): p. 237-246.
31. Higgins, D.A., et al., *The major Vibrio cholerae autoinducer and its role in virulence factor production*. Nature, 2007. **450**(7171): p. 883-886.

32. Ruby, E.G., *Lessons from a cooperative, bacterial-animal association: the Vibrio fischeri-Euprymna scolopes light organ symbiosis*. Annu Rev Microbiol, 1996. **50**: p. 591-624.
33. Zhao, K., et al., *Genes as Early Responders Regulate Quorum-Sensing and Control Bacterial Cooperation in Pseudomonas aeruginosa*. PLoS ONE, 2014. **9**(7): p. e101887.
34. De Keersmaecker, S.C.J., K. Sonck, and J. Vanderleyden, *Let LuxS speak up in AI-2 signaling*. Trends in Microbiology, 2006. **14**(3): p. 114-119.
35. Bassler, B.L., *How bacteria talk to each other: regulation of gene expression by quorum sensing*. Current Opinion in Microbiology, 1999. **2**(6): p. 582-587.
36. Schauder, S., et al., *The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule*. Mol Microbiol, 2001. **41**(2): p. 463-76.
37. Otto, M., *Quorum-sensing control in Staphylococci – a target for antimicrobial drug therapy?* FEMS Microbiology Letters, 2004. **241**(2): p. 135-141.
38. Defoirdt, T., G. Brackman, and T. Coenye, *Quorum sensing inhibitors: how strong is the evidence?* Trends in Microbiology, 2013. **21**(12): p. 619-624.
39. Zhu, J. and G.F. Kaufmann, *Quo vadis quorum quenching?* Current Opinion in Pharmacology, 2013. **13**(5): p. 688-698.
40. Hammer, B.K. and B.L. Bassler, *Quorum sensing controls biofilm formation in Vibrio cholerae*. Mol Microbiol, 2003. **50**(1): p. 101-4.
41. Stonehouse, E., et al., *Integration host factor positively regulates virulence gene expression in Vibrio cholerae*. J Bacteriol, 2008. **190**(13): p. 4736-48.
42. Miller, M.B., et al., *Parallel quorum sensing systems converge to regulate virulence in Vibrio cholerae*. Cell, 2002. **110**(3): p. 303-14.
43. Lenz, D.H. and B.L. Bassler, *The small nucleoid protein Fis is involved in Vibrio cholerae quorum sensing*. Mol Microbiol, 2007. **63**(3): p. 859-71.
44. Lenz, D.H., et al., *CsrA and three redundant small RNAs regulate quorum sensing in Vibrio cholerae*. Mol Microbiol, 2005. **58**(4): p. 1186-202.
45. Miller, M.B., et al., *Parallel quorum sensing systems converge to regulate virulence in Vibrio cholerae*. Cell, 2002. **110**(3): p. 303-314.
46. Hunter, G.A. and J.P. Keener, *Mechanisms underlying the additive and redundant Qrr phenotypes in Vibrio harveyi and Vibrio cholerae*. J Theor Biol, 2014. **340**: p. 38-49.
47. Ng, W.L., et al., *Signal production and detection specificity in Vibrio CqsA/CqsS quorum-sensing systems*. Mol Microbiol, 2011. **79**(6): p. 1407-17.
48. Jang, J., et al., *Regulation of hemagglutinin/protease expression by the VarS/VarA-CsrA/B/C/D system in Vibrio cholerae*. Microb Pathog, 2010. **48**(6): p. 245-50.
49. Tu, K.C., et al., *Negative feedback loops involving small regulatory RNAs precisely control the Vibrio harveyi quorum-sensing response*. Mol Cell, 2010. **37**(4): p. 567-79.
50. Svenningsen, S.L., K.C. Tu, and B.L. Bassler, *Gene dosage compensation calibrates four regulatory RNAs to control Vibrio cholerae quorum sensing*. Embo j, 2009. **28**(4): p. 429-39.
51. Lin, W., G. Kovacikova, and K. Skorupski, *Requirements for Vibrio cholerae HapR binding and transcriptional repression at the hapR promoter are distinct from those at the aphA promoter*. J Bacteriol, 2005. **187**(9): p. 3013-9.

52. Svenningsen, S.L., C.M. Waters, and B.L. Bassler, *A negative feedback loop involving small RNAs accelerates Vibrio cholerae's transition out of quorum-sensing mode*. *Genes Dev*, 2008. **22**(2): p. 226-38.
53. Ramos, J.L., et al., *The TetR family of transcriptional repressors*. *Microbiol Mol Biol Rev*, 2005. **69**(2): p. 326-56.
54. Jobling, M.G. and R.K. Holmes, *Characterization of hapR, a positive regulator of the Vibrio cholerae HA/protease gene hap, and its identification as a functional homologue of the Vibrio harveyi luxR gene*. *Mol Microbiol*, 1997. **26**(5): p. 1023-34.
55. Ng, W.-L., et al., *Probing bacterial transmembrane histidine kinase receptor-ligand interactions with natural and synthetic molecules*. *Proceedings of the National Academy of Sciences*, 2010. **107**(12): p. 5575-5580.
56. Bolitho, M.E., et al., *Small molecule probes of the receptor binding site in the Vibrio cholerae CAI-1 quorum sensing circuit*. *Bioorg Med Chem*, 2011. **19**(22): p. 6906-18.
57. Lowery, C.A., et al., *Quorum sensing in Vibrio harveyi: probing the specificity of the LuxP binding site*. *Bioorg Med Chem Lett*, 2005. **15**(9): p. 2395-8.
58. Rui, F., et al., *Stereochemical diversity of AI-2 analogs modulates quorum sensing in Vibrio harveyi and Escherichia coli*. *Bioorg Med Chem*, 2012. **20**(1): p. 249-56.
59. Stock, A.M., V.L. Robinson, and P.N. Goudreau, *Two-component signal transduction*. *Annu Rev Biochem*, 2000. **69**: p. 183-215.
60. Wei, Y., et al., *Ligand and antagonist driven regulation of the Vibrio cholerae quorum-sensing receptor CqsS*. *Mol Microbiol*, 2012. **83**(6): p. 1095-108.
61. Neiditch, M.B., et al., *Regulation of LuxPQ receptor activity by the quorum-sensing signal autoinducer-2*. *Mol Cell*, 2005. **18**(5): p. 507-18.
62. Neiditch, M.B., et al., *Ligand-induced asymmetry in histidine sensor kinase complex regulates quorum sensing*. *Cell*, 2006. **126**(6): p. 1095-108.
63. Freeman, J.A. and B.L. Bassler, *Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in Vibrio harveyi*. *J Bacteriol*, 1999. **181**(3): p. 899-906.
64. Bassler, B.L., M. Wright, and M.R. Silverman, *Sequence and function of LuxO, a negative regulator of luminescence in Vibrio harveyi*. *Mol Microbiol*, 1994. **12**(3): p. 403-12.
65. Freeman, J.A. and B.L. Bassler, *A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in Vibrio harveyi*. *Mol Microbiol*, 1999. **31**(2): p. 665-77.
66. Lenz, D.H., et al., *The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in Vibrio harveyi and Vibrio cholerae*. *Cell*, 2004. **118**(1): p. 69-82.
67. Vogel, J. and B.F. Luisi, *Hfq and its constellation of RNA*. *Nat Rev Microbiol*, 2011. **9**(8): p. 578-89.
68. Masse, E., F.E. Escorcia, and S. Gottesman, *Coupled degradation of a small regulatory RNA and its mRNA targets in Escherichia coli*. *Genes Dev*, 2003. **17**(19): p. 2374-83.
69. Fenley, A.T., S.K. Banik, and R.V. Kulkarni, *Computational modeling of differences in the quorum sensing induced luminescence phenotypes of Vibrio harveyi and Vibrio cholerae*. *J Theor Biol*, 2011. **274**(1): p. 145-53.

70. Hunter, G.A., F.G. Vasquez, and J.P. Keener, *A mathematical model and quantitative comparison of the small RNA circuit in the Vibrio harveyi and Vibrio cholerae quorum sensing systems*. Phys Biol, 2013. **10**(4): p. 046007.
71. Svenningsen, S.L., C.M. Waters, and B.L. Bassler, *A negative feedback loop involving small RNAs accelerates Vibrio cholerae's transition out of quorum-sensing mode*. Genes & Development, 2008. **22**(2): p. 226-238.
72. De Silva, R.S., et al., *Crystal structure of the Vibrio cholerae quorum-sensing regulatory protein HapR*. Journal of Bacteriology, 2007. **189**(15): p. 5683-5691.
73. Kovacikova, G. and K. Skorupski, *Regulation of virulence gene expression in Vibrio cholerae by quorum sensing: HapR functions at the aphA promoter*. Mol Microbiol, 2002. **46**(4): p. 1135-47.
74. Czaplewski, L., et al., *Alternatives to antibiotics-a pipeline portfolio review*. Lancet Infect Dis, 2016. **16**(2): p. 239-51.
75. Cotter, P.D., R.P. Ross, and C. Hill, *Bacteriocins - a viable alternative to antibiotics?* Nat Rev Microbiol, 2013. **11**(2): p. 95-105.
76. Balciunas, E.M., et al., *Novel biotechnological applications of bacteriocins: A review*. Food control, 2013. **32**(1): p. 134-142.
77. Duquesne, S., et al., *Microcins, gene-encoded antibacterial peptides from enterobacteria*. Nat Prod Rep, 2007. **24**(4): p. 708-34.
78. Gillor, O., B.C. Kirkup, and M.A. Riley, *Colicins and microcins: the next generation antimicrobials*. Adv Appl Microbiol, 2004. **54**: p. 129-46.
79. Dobson, A., et al., *Bacteriocin production: a probiotic trait?* Applied and Environmental Microbiology, 2012. **78**(1): p. 1-6.
80. Hwang, I.Y., et al., *Reprogramming Microbes to Be Pathogen-Seeking Killers*. ACS Synthetic Biology, 2013. **3**(4): p. 228-237.
81. O'Shea, E.F., et al., *Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid*. Int J Food Microbiol, 2012. **152**(3): p. 189-205.
82. Zschüttig, A., et al., *Identification and Characterization of Microcin S, a New Antibacterial Peptide Produced by Probiotic Escherichia coli G3/10*. PLoS ONE, 2012. **7**(3): p. e33351.
83. Rebuffat, S., *Microcins in action: amazing defence strategies of Enterobacteria*. Biochem Soc Trans, 2012. **40**(6): p. 1456-62.
84. Pons, A.M., et al., *Genetic analysis and complete primary structure of microcin L*. Antimicrob Agents Chemother, 2004. **48**(2): p. 505-13.
85. Young, R., I.-N. Wang, and W.D. Roof, *Phages will out: strategies of host cell lysis*. Trends in Microbiology. **8**(3): p. 120-128.
86. Schmelcher, M., D.M. Donovan, and M.J. Loessner, *Bacteriophage endolysins as novel antimicrobials*. Future Microbiol, 2012. **7**(10): p. 1147-71.
87. Briers, Y., et al., *Engineered Endolysin-Based "Artilylins" To Combat Multidrug-Resistant Gram-Negative Pathogens*. mBio, 2014. **5**(4).
88. Gerstmans, H., et al., *From endolysins to Artilylin®s: novel enzyme-based approaches to kill drug-resistant bacteria*. Biochemical Society Transactions, 2016. **44**(1): p. 123-128.
89. Briers, Y., et al., *Art-175 Is a Highly Efficient Antibacterial against Multidrug-Resistant Strains and Persists of Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy, 2014. **58**(7): p. 3774-3784.

90. Defraigne, V., et al., *Efficacy of Artilysin Art-175 against Resistant and Persistent Acinetobacter baumannii*. Antimicrobial Agents and Chemotherapy, 2016. **60**(6): p. 3480-3488.
91. Skerlavaj, B., et al., *SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes*. FEBS Letters, 1999. **463**(1-2): p. 58-62.
92. Jayawardene, A. and H. Farkas-Himsley, *Mode of action of vibriocin*. J Bacteriol, 1970. **102**(2): p. 382-8.
93. Moghaddam, M.M., et al., *Comparison of in vitro antibacterial activities of two cationic peptides CM15 and CM11 against five pathogenic bacteria: Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio cholerae, Acinetobacter baumannii, and Escherichia coli*. Probiotics and Antimicrobial Proteins, 2012. **4**(2): p. 133-139.
94. da Silva, A.P.G., et al., *In vitro and in vivo antimicrobial activity of granulysin-derived peptides against Vibrio cholerae*. Journal of Antimicrobial Chemotherapy, 2008. **61**(5): p. 1103-1109.
95. Velusamy, V., et al., *An overview of foodborne pathogen detection: in the perspective of biosensors*. Biotechnology advances, 2010. **28**(2): p. 232-254.
96. Yang, Z. and C. Zhang, *Single-enzyme nanoparticles based urea biosensor*. Sensors and Actuators B: Chemical, 2013. **188**: p. 313-317.
97. Kim, G., et al., *A microfluidic nano-biosensor for the detection of pathogenic Salmonella*. Biosensors and Bioelectronics, 2015(0).
98. Yu, C.Y., et al., *Dry-reagent gold nanoparticle-based lateral flow biosensor for the simultaneous detection of Vibrio cholerae serogroups O1 and O139*. Journal of Microbiological Methods, 2011. **86**(3): p. 277-282.
99. Waswa, J., J. Irudayaraj, and C. DebRoy, *Direct detection of E. Coli O157:H7 in selected food systems by a surface plasmon resonance biosensor*. LWT - Food Science and Technology, 2007. **40**(2): p. 187-192.
100. Chen, S.-H., et al., *Using oligonucleotide-functionalized Au nanoparticles to rapidly detect foodborne pathogens on a piezoelectric biosensor*. Journal of Microbiological Methods, 2008. **73**(1): p. 7-17.
101. Chua, A., et al., *A rapid DNA biosensor for the molecular diagnosis of infectious disease*. Biosensors and Bioelectronics, 2011. **26**(9): p. 3825-3831.
102. Tichoniuk, M., et al., *Electrochemical detection of foodborne pathogen Aeromonas hydrophila by DNA hybridization biosensor*. Biosensors and Bioelectronics, 2010. **26**(4): p. 1618-1623.
103. Zhang, J., et al., *Rapid detection of several foodborne pathogens by FOF1-ATPase molecular motor biosensor*. Journal of Microbiological Methods, 2013. **93**(1): p. 37-41.
104. Banerjee, P. and A.K. Bhunia, *Cell-based biosensor for rapid screening of pathogens and toxins*. Biosensors and Bioelectronics, 2010. **26**(1): p. 99-106.
105. Deng, X., et al., *Construction of a dual fluorescence whole-cell biosensor to detect N-acyl homoserine lactones*. Journal of Environmental Sciences, 2014. **26**(2): p. 415-422.
106. Saeidi, N., et al., *Engineering microbes to sense and eradicate Pseudomonas aeruginosa, a human pathogen*. Mol Syst Biol, 2011. **7**: p. 521.
107. Pires, A.C.d.S., et al., *A colorimetric biosensor for the detection of foodborne bacteria*. Sensors and Actuators B: Chemical, 2011. **153**(1): p. 17-23.
108. Way, J.C., et al., *Integrating biological redesign: where synthetic biology came from and where it needs to go*. Cell, 2014. **157**(1): p. 151-61.

109. Endy, D., P. Lincoln, and R. Murray, *2003 Synthetic Biology Study*. 2003.
110. T.F., K., *Idempotent vector design for standard assembly of BioBricks*. MIT Synthetic Biology Working Group Technical Reports, 2003.
111. igem. Available from: <http://parts.igem.org/Catalog>.
112. Voigt, C.A., *Genetic parts to program bacteria*. Current Opinion in Biotechnology, 2006. **17**(5): p. 548-557.
113. Mutalik, V.K., et al., *Quantitative estimation of activity and quality for collections of functional genetic elements*. Nat Methods, 2013. **10**(4): p. 347-53.
114. contributors, O. *BglBrick*. 29 September 2009 8 September 2014]; Available from: <http://openwetware.org/index.php?title=BglBrick&oldid=354679>.
115. Lee, T.S., et al., *BglBrick vectors and datasheets: A synthetic biology platform for gene expression*. J Biol Eng, 2011. **5**: p. 12.
116. Brophy, J.A.N. and C.A. Voigt, *Principles of genetic circuit design*. Nat Meth, 2014. **11**(5): p. 508-520.
117. Ramalingam, K.I., et al., *Forward engineering of synthetic bio-logical AND gates*. Biochemical engineering journal, 2009. **47**(1-3): p. 38-47.
118. Wang, B., et al., *Engineering modular and orthogonal genetic logic gates for robust digital-like synthetic biology*. Nat Commun, 2011. **2**: p. 508.
119. Gardner, T.S., C.R. Cantor, and J.J. Collins, *Construction of a genetic toggle switch in Escherichia coli*. Nature, 2000. **403**(6767): p. 339-342.
120. Ajo-Franklin, C.M., et al., *Rational design of memory in eukaryotic cells*. Genes & Development, 2007. **21**(18): p. 2271-2276.
121. Bonnet, J., P. Subsoontorn, and D. Endy, *Rewritable digital data storage in live cells via engineered control of recombination directionality*. Proceedings of the National Academy of Sciences, 2012. **109**(23): p. 8884-8889.
122. Elowitz, M.B. and S. Leibler, *A synthetic oscillatory network of transcriptional regulators*. Nature, 2000. **403**(6767): p. 335-338.
123. Tinberg, C.E., et al., *Computational design of ligand-binding proteins with high affinity and selectivity*. Nature, 2013. **501**(7466): p. 212-216.
124. Ranganathan, S., P.F. Suthers, and C.D. Maranas, *OptForce: An Optimization Procedure for Identifying All Genetic Manipulations Leading to Targeted Overproductions*. PLoS Comput Biol, 2010. **6**(4): p. e1000744.
125. Salis, H.M., *The ribosome binding site calculator*. Methods Enzymol, 2011. **498**: p. 19-42.
126. Nielsen, A.A., et al., *Genetic circuit design automation*. Science, 2016. **352**(6281): p. aac7341.
127. Kobayashi, H., et al., *Programmable cells: Interfacing natural and engineered gene networks*. Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(22): p. 8414-8419.
128. Keasling, J.D., *Synthetic biology and the development of tools for metabolic engineering*. Metabolic Engineering, 2012. **14**(3): p. 189-195.
129. Anesiadis, N., et al., *Analysis and Design of a Genetic Circuit for Dynamic Metabolic Engineering*. ACS Synthetic Biology, 2013. **2**(8): p. 442-452.
130. Yoo, S.M., D. Na, and S.Y. Lee, *Design and use of synthetic regulatory small RNAs to control gene expression in Escherichia coli*. Nat. Protocols, 2013. **8**(9): p. 1694-1707.
131. Cong, L., et al., *Multiplex Genome Engineering Using CRISPR/Cas Systems*. Science, 2013. **339**(6121): p. 819-823.

132. Marraffini, L.A. and E.J. Sontheimer, *CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea*. Nat Rev Genet, 2010. **11**(3): p. 181-190.
133. Callura, J.M., et al., *Tracking, tuning, and terminating microbial physiology using synthetic riboregulators*. Proc Natl Acad Sci U S A, 2010. **107**(36): p. 15898-903.
134. Nudler, E. and A.S. Mironov, *The riboswitch control of bacterial metabolism*. Trends Biochem Sci, 2004. **29**(1): p. 11-7.
135. Glass, J.I., et al., *Essential genes of a minimal bacterium*. Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(2): p. 425-430.
136. Delebecque, C.J., et al., *Organization of Intracellular Reactions with Rationally Designed RNA Assemblies*. Science, 2011. **333**(6041): p. 470-474.
137. Steen, E.J., et al., *Microbial production of fatty-acid-derived fuels and chemicals from plant biomass*. Nature, 2010. **463**(7280): p. 559-562.
138. Moon, T.S., et al., *Production of glucaric acid from a synthetic pathway in recombinant Escherichia coli*. Applied and Environmental Microbiology, 2009. **75**(3): p. 589-595.
139. Na, D., et al., *Metabolic engineering of Escherichia coli using synthetic small regulatory RNAs*. Nat Biotech, 2013. **31**(2): p. 170-174.
140. Zhang, F. and J. Keasling, *Biosensors and their applications in microbial metabolic engineering*. Trends in Microbiology. **19**(7): p. 323-329.
141. Carlson, R., *The changing economics of DNA synthesis*. Nat Biotech, 2009. **27**(12): p. 1091-1094.
142. Barrangou, R., et al., *CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes*. Science, 2007. **315**(5819): p. 1709-1712.
143. Wiedenheft, B., et al., *Structures of the RNA-guided surveillance complex from a bacterial immune system*. Nature, 2011. **477**(7365): p. 486-489.
144. Cong, L., et al., *Multiplex genome engineering using CRISPR/Cas systems*. Science (New York), 2013. **339**(6121): p. 819-823.
145. Wiedenheft, B., S.H. Sternberg, and J.A. Doudna, *RNA-guided genetic silencing systems in bacteria and archaea*. Nature, 2012. **482**(7385): p. 331-338.
146. Mali, P., K.M. Esvelt, and G.M. Church, *Cas9 as a versatile tool for engineering biology*. Nature Methods, 2013. **10**(10): p. 957-963.
147. Cress, B.F., et al., *CRISPathBrick: Modular Combinatorial Assembly of Type II-A CRISPR Arrays for dCas9-Mediated Multiplex Transcriptional Repression in E. coli*. ACS synthetic biology [electronic resource], 2015. **4**(9): p. 987-1000.
148. Gasiunas, G., et al., *Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(39): p. E2579-E2586.
149. Zetsche, B., et al., *Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system*. Cell, 2015. **163**(3): p. 759-771.
150. Anders, C., et al., *Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease*. Nature, 2014. **513**(7519): p. 569-573.
151. Qi, L.S., et al., *Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression*. Cell, 2013. **152**(5): p. 1173-1183.
152. Gilbert, Luke A., et al., *Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation*. Cell, 2014. **159**(3): p. 647-661.

153. Ji, W., et al., *Specific gene repression by CRISPRi system transferred through bacterial conjugation*. ACS synthetic biology [electronic resource], 2014. **3**(12): p. 929-931.
154. Larson, M.H., et al., *CRISPR interference (CRISPRi) for sequence-specific control of gene expression*. Nature Protocols, 2013. **8**(11): p. 2180-2196.
155. Rath, D., et al., *Efficient programmable gene silencing by Cascade*. Nucleic Acids Research, 2014.
156. Didovyk, A., et al., *Orthogonal Modular Gene Repression in Escherichia coli Using Engineered CRISPR/Cas9*. ACS synthetic biology [electronic resource], 2016. **5**(1): p. 81-88.
157. Doench, J.G., et al., *Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation*. Nature Biotechnology, 2014. **32**(12): p. 1262-1267.
158. Hsu, P.D., et al., *DNA targeting specificity of RNA-guided Cas9 nucleases*. Nature Biotechnology, 2013. **31**(9): p. 827-832.
159. Xu, H., et al., *Sequence determinants of improved CRISPR sgRNA design*. Genome Research, 2015. **25**(8): p. 1147-1157.
160. Mali, P., et al., *CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering*. Nature Biotechnology, 2013. **31**(9): p. 833-838.
161. Peters, J.M., et al., *Bacterial CRISPR: accomplishments and prospects*. Current Opinion in Microbiology, 2015. **27**: p. 121-126.
162. Zhang, F., *CRISPR-Cas9: Prospects and Challenges*. Hum Gene Ther, 2015. **26**(7): p. 409-10.
163. Wang, T., et al., *Genetic screens in human cells using the CRISPR-Cas9 system*. Science, 2014. **343**(6166): p. 80-4.
164. Stanton, B.C., et al., *Genomic mining of prokaryotic repressors for orthogonal logic gates*. Nature Chemical Biology, 2014. **10**(2): p. 99-105.
165. Cormack, B.P., R.H. Valdivia, and S. Falkow, *FACS-optimized mutants of the green fluorescent protein (GFP)*. Gene, 1996. **173**(1 Spec No): p. 33-38.
166. Freeman, J.A. and B.L. Bassler, *A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in Vibrio harveyi*. Molecular Microbiology, 1999. **31**(2): p. 665-677.
167. Holowko, M.B., et al., *Biosensing Vibrio cholerae with Genetically Engineered Escherichia coli*. ACS Synthetic Biology, 2016. **5**(11): p. 1275-1283.
168. Morett, E. and L. Segovia, *The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains*. Journal of Bacteriology, 1993. **175**(19): p. 6067-6074.
169. Zhang, X., et al., *Kinetic properties of rrn promoters in Escherichia coli*. Biochimie, 2002. **84**(10): p. 981-996.
170. Mukhopadhyay, A.K., et al., *Distribution and virulence of Vibrio cholerae belonging to serogroups other than O1 and O139: a nationwide survey*. Epidemiology and Infection, 1995. **114**(1): p. 65-70.
171. *Promoters/Catalog/Anderson - parts.igem.org*.
172. *Ribosome Binding Sites/Prokaryotic/Constitutive/Community Collection - parts.igem.org*.
173. Wagner, S., et al., *Consequences of membrane protein overexpression in Escherichia coli*. Molecular & Cellular Proteomics, 2007. **6**(9): p. 1527-1550.
174. Salis, H.M., *The ribosome binding site calculator*. Methods in Enzymology, 2011. **498**: p. 19-42.

175. Goldbeter, A. and D.E. Koshland, *An amplified sensitivity arising from covalent modification in biological systems*. Proceedings of the National Academy of Sciences of the United States of America, 1981. **78**(11): p. 6840-6844.
176. Chou, I.C. and E.O. Voit, *Recent developments in parameter estimation and structure identification of biochemical and genomic systems*. Mathematical Biosciences, 2009. **219**(2): p. 57-83.
177. Cecchini, F., et al., *Vibrio cholerae detection: Traditional assays, novel diagnostic techniques and biosensors*. TrAC Trends in Analytical Chemistry, 2016. **79**: p. 199-209.
178. Repaske, R., *Lysis of gram-negative organisms and the role of versene*. Biochimica et Biophysica Acta, 1958. **30**(2): p. 225-232.
179. Pasotti, L., et al., *Characterization of a synthetic bacterial self-destruction device for programmed cell death and for recombinant proteins release*. Journal of Biological Engineering, 2011. **5**: p. 8-8.
180. Brophy, J.A.N. and C.A. Voigt, *Principles of genetic circuit design*. Nature Methods, 2014. **11**(5): p. 508-520.
181. Schultz, M., *Clinical use of E. coli Nissle 1917 in inflammatory bowel disease*. Inflamm Bowel Dis., 2008. **14**(7): p. 1012-8.
182. Grozdanov, L., et al., *Analysis of the genome structure of the nonpathogenic probiotic Escherichia coli strain Nissle 1917*. Journal of Bacteriology, 2004. **186**(16): p. 5432-5441.
183. Kruis, W., et al., *A double-blind placebo-controlled trial to study therapeutic effects of probiotic Escherichia coli Nissle 1917 in subgroups of patients with irritable bowel syndrome*. Int J Colorectal Dis, 2012. **27**(4): p. 467-74.
184. Loessner, H., et al., *Drug-inducible remote control of gene expression by probiotic Escherichia coli Nissle 1917 in intestine, tumor and gall bladder of mice*. Microbes and Infection, 2009. **11**(14-15): p. 1097-1105.
185. Sambrook, J., *Molecular cloning : a laboratory manual / Joseph Sambrook, David W. Russell*, ed. D.W. Russell and L. Cold Spring Harbor. 2001, Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory.
186. Gibson, D.G., et al., *Enzymatic assembly of DNA molecules up to several hundred kilobases*. Nature Methods, 2009. **6**(5): p. 343-345.
187. Benson, D.A., et al., *GenBank*. Nucleic Acids Research, 2005. **33**(Database issue): p. D34-D38.
188. Lee, T.S., et al., *BglBrick vectors and datasheets: A synthetic biology platform for gene expression*. Journal of Biological Engineering, 2011. **5**: p. 12.